THE L FORMS OF BACTERIA[1]

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In 1935 Klieneberger (46) isolated from cultures of *Streptobacillus moniliformis* a strange organism which she designated as L1. This organism differed in many respects from bacteria and resembled the organisms of bovine pleuropneumonia. The morphology of the latter is so different from bacteria that it is classified in a different order or even in an independent class (65, 75). A few years later, Dienes (9) reported the development of tiny colonies similar to the L1 in occasional cultures of various species, and subsequently he isolated them from a *Flavobacterium* (9) and from a *Bacteroides* strain (12). Pierce (71) observed in 1942 that the L1 is resistant to penicillin, and subsequently organisms similar to L1 were isolated from many gram negative and gram positive bacteria, by cultivating bacteria in the presence of penicillin. Development of similar forms occurs also in various other conditions such as spontaneous autolysis and exposure of bacteria to certain bacteriostatic chemicals, to phage, and to antibodies (25, 33).

For a long time Klieneberger (46, 51) supported the hypothesis that the L1 and the bacillus were unrelated organisms living in symbiosis. All other investigators (4, 6, 8, 41, 67) who studied the L1 concluded that it was a growth form of the bacillus. This viewpoint is now generally held since Klieneberger (52) recently abandoned the symbiosis theory and accepted the identity of the L1 and *Streptobacillus moniliformis*. Apparently the bacteria undergo a strange transformation in response to various influences, and they survive and multiply as tiny soft forms often unrecognizable with the usual bacteriological methods. The study of these forms is in a preliminary stage and their significance in the life and activity of bacteria is not known. However, the discovery of these forms presents new and significant problems for the study of bacteria.

This review will be introduced by a short consideration of the technical procedures necessary for the study of L forms. This will be followed by a description of the observations made in different groups of bacteria. The second half of the review contains a survey of their properties, their possible nature and significance and their relationship to the pleuropneumonia group of organisms. A purely descriptive nomenclature will be used. The peculiar forms which are the subject of this review, when isolated from bacteria, are called "L forms" and their colonies and cultures are referred to as "L type." The L1 is the L form of *Streptobacillus moniliformis*. The exact meaning of these terms will become clear in the descriptions of the observations. Similar forms found as independent organisms without apparent relationship to any known bacterium are designated as organisms of the pleuropneumonia group or pleuropneumonia-like organisms after the

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agent of bovine pleuropneumonia, which was the first organism to be recognized in this group. In the description of the morphology of L type cultures the terms "small forms," "swollen forms," and "large bodies" are employed. The latter two terms will be applied also to pleomorphic forms of bacteria.

TECHNICAL PROCEDURES

The L forms and the pleuropneumonia group of organisms which they resemble are soft and fragile. Smears and impression preparations rarely show their true forms and are inappropriate for their study. The derivation of the different morphological elements of the cultures and also the derivation of the L forms from the bacteria can be recognized, as in the study of fungi, only by following step by step the development of the cultures. The first detailed observations on the morphology of the organisms of bovine pleuropneumonia were made with darkfield examination of broth cultures (2). Oerskov (64, 65) followed the development of the cultures on thin transparent agar with oil immersion. These simple methods are not sufficient for the study of the L forms. In many cases they do not grow in liquid media and their growth from bacteria was never observed in such media. Restriction to cultures especially grown on transparent agar excludes from examination routine bacteriological cultures in which most of the L forms were discovered and restricts experimentation with the media. Furthermore, stained preparations give more information about the morphology of the organisms.

The usefulness of direct microscopic examination of agar cultures is increased considerably when combined with appropriate staining (8, 16). Certain dyes color bacterial organisms deeply while the medium is only slightly stained. Methylene blue and azure are especially suitable because they do not form precipitates and the bright blue color differentiates the intact bacterial structures from autolyzed organisms and from impurities in the agar. Distortion of the cultures during staining can be avoided by drying the staining solution on coverslips, as in vital staining, and by careful placing of the coverslips on the agar. In these preparations the entire growth on the agar is seen, undisturbed in its natural position. The plates can be examined macroscopically and microscopically at the same time, and this can be repeated at short intervals. Turbid media including blood agar plates can be studied also. The reviewers' success in discovering L forms in many bacterial cultures and in isolating pleuropneumonia-like organisms from patients was the result of quick and efficient microscopic examination of the agar cultures, made possible by the stained agar preparations.

The techniques used to make these preparations and to make dry preparations have been described (8, 32). A more detailed description of the techniques than is available in this publication may be obtained from the reviewers.

Direct staining of agar cultures with staining solution has been suggested (4, 66, 78). In certain instances this gives excellent results, but for most purposes it cannot be used. The bacterial colonies are broken up, the bacteria are distributed on the agar, and the surface growth of L type colonies is usually completely destroyed.

Klieneberger (45, 54) endeavored to overcome the difficulties inherent in the study of the pleuropneumonia group by improving the technique of impression preparations. This consisted of the fixation of an agar block inverted on a coverslip; the principle of this method was devised by Kuhn (57). The lifting of the agar leaves an excellent impression of the culture on the coverslip. The morphology of the individual organism is most clearly apparent in such preparations. Not only is the staining better than in the wet stained agar preparations, but the organisms are seen in one plane and can be stained by a variety of methods. In stained agar preparations, even the smallest pleuropneumonia-like colonies are tridimensional and only a few organisms can be seen simultaneously. A great disadvantage of the agar fixation method, however, is that the preparations do not contain the entire growth developing on the plates, and for this reason certain growth processes cannot be observed on them. Autolyzed organisms are poorly if at all visible. Artefacts which are difficult to interpret are often produced. Furthermore, the making of the preparations requires many hours and they cannot be used for simultaneous macroscopical and microscopical study of the cultures. Although the method made it possible for Klieneberger to discover the L1 and to pioneer in the study of the pleuropneumonia group, it was not appropriate for the discovery of tiny L type colonies which develop in many other bacterial cultures.

Some of the more recently developed microscopic techniques also have been employed in the study of the L forms. Electron microscopy (82) was used by Smith, Hillier and Mudd. Klieneberger and Smiles (55) used "oblique incident illumination." The appearance of the colonies resembled that seen by dark field. The method can be applied only to cultures grown in a special slide. With phase microscopy also only cultures grown on thin transparent agar can be examined. The morphology of small L type colonies is clearly visible but the resolution of large colonies is poorer than in stained preparations. Phase microscopy is excellent for the observation of certain growth phenomena but it cannot replace the stained agar preparations.

In most instances L type cultures cannot be transferred with a loop. Klieneberger (46) subcultured the L1 by cutting out a square of agar containing the colonies and streaking it on fresh medium. The agar block is left on the medium because growth often develops only beneath it. To isolate L type colonies occurring in mixed growth with bacteria, pieces of agar containing only the L type colonies are cut out and are transferred to fresh media. Sometimes growth can be obtained in a mixed culture by touching an L colony with a loop and making a streak on the agar without lifting the loop.

THE DEVELOPMENT AND PROPERTIES OF L FORMS IN VARIOUS BACTERIAL SPECIES

Streptobacillus moniliformis. Pleomorphism and variability of morphological appearance is characteristic of this species. The colonies may consist of short, regularly shaped bacilli, or of segmented or unsegmented filaments. The filaments may become twisted and fragmented into small granules. All these various

forms may swell to large round bodies, and sometimes 24-hour-old colonies consist exclusively of large bodies. Autolysis is pronounced in the pleomorphic cultures. The filaments were thought to show true branching and the organism was classified with the actinomycetes (84). When examined with appropriate methods, however, branching is not observed (8, 46, 91). The organism is a common saprophyte in the pharynx of rats and as Van Rooyen (91) pointed out, it is reasonable to classify it with the hemophilic bacilli.

Well spaced colonies of *Streptobacillus moniliformis* grow on blood or serum plates in 24 hours to a diameter of 1 to 2 mm. The L1 appear as pinpoint colonies among the bacterial colonies and grow in the course of a few days to a diameter of 0.1 mm, or somewhat larger. They are produced in variable number by different strains, the more pleomorphic producing more than the less pleomorphic ones. Examined with the hand lens the L1 colonies appear rough. They cannot be removed from the agar with a loop. With the low power of the microscope a large part of the colonies is found beneath the surface of the medium as a dense spherical mass. The surface of the colony extends further on the medium and consists of large round bodies and of empty round blebs produced by autolysis of the large bodies.

The origin of L1 colonies, their morphology and their relationship to the bacilli remained controversial for a long time. Klieneberger, in the years following the discovery of the L1, made many pertinent observations. She observed that all strains of *S. moniliformis* produce L1 colonies, and it was not possible to eliminate the L1 from the bacillary cultures. Both the bacillus and L1 agglutinated with specific sera produced with either organism. She observed the reappearance of the bacilli when the cultures of L1 were transferred to broth, and also that they became stabilized in the L1 form after a long period of cultivation on solid media.

Klieneberger's views on the morphology of L1 and *S. moniliformis* were modified several times. Her latest description of the morphology of the L1 is as follows: The fundamental reproductive units are small "elementary corpuscles" containing a chromatin granule with a small amount of protoplasm. The corpuscles multiply by fission or swell to larger forms up to several microns in diameter which are produced by reproduction of the granules inside a common envelope. The large forms may segment into the corpuscles at any stage of their development. All elements of the culture are soft and malleable and do not have a rigid cell-wall like the bacteria. Klieneberger believed that in cultures of *S. moniliformis* the pleomorphic forms, especially the large bodies, do not derive from the bacilli but are forms of the symbiont, L1, and she identified the small granules visible in autolyzed bacillary cultures with the elementary corpuscles of L1.

It seemed to Klieneberger unlikely that an organism with the properties of L1 can be a variant of a bacterium. The observation that the L1 after long cultivation fails to return to bacillary form supported this impression. The return of bacilli in young L1 cultures and the serological similarity of the L1 and of the bacilli were explained by the supposition that the two organisms grow in mixed cultures. The cytological study of various bacteria later convinced Klieneberger that the morphological difference between the L1 and bacillus is less significant

than she originally believed. These studies and the accumulation of observations on L transformation in other species induced Klieneberger to accept the genetical identity of L1 and the bacillus (52).

Dienes, like Klieneberger, studied *S. moniliformis*, L1 and the pleuropneumonia-like organisms simultaneously (8, 16, 20). In addition, he studied a large number of freshly isolated pleomorphic bacteria. His first impression was, and has remained unchanged, that the morphology of these organisms, though different in details, is essentially similar. The small granules in the cultures of L1 and of the pleuropneumonia-like organisms are stained with methylene blue like bacteria, and sometimes appear as perfectly shaped, small, bipolar staining bacilli. In young colonies, some grow as short filaments, and the pleomorphism of the cultures is produced by swelling of the organisms into large bodies of various size, as in bacterial cultures. The morphology of these organisms seems essentially similar to the morphology of pleomorphic bacilli.

Observing the development of *S. moniliformis* cultures on agar, it became apparent that the bacterial colonies do not contain two types of growing elements. The large bodies are produced by swelling and the granules by fractionation of the bacillary filaments as in other pleomorphic cultures. Neither the large bodies nor the granules show any multiplication in the bacterial colonies. Dienes never found that the granules, which Klieneberger identified in autolyzed cultures (50) as the elementary corpuscles of L1, were viable. L1 colonies develop exclusively from the large bodies (13) when bacillary cultures are transplanted. These bodies increase in size, their contours become slightly irregular and from one or from several points strands of L1 granules grow into the agar. The appearance of germinating large bodies is very characteristic. Such structures can be seen only in young cultures of the L type or of the pleuropneumonia-like organisms, and their presence is sufficient to indicate the development of these organisms. Not all large bodies develop into L1, but some reproduce bacilli of the usual morphology (14). The bacilli develop inside of the large bodies. Dienes observed also the return of the bacilli in broth cultures of L1 (8).

The observations that the morphology of L1 is not essentially different from that of bacteria, that the L1 derives from the bacilli and returns into bacilli, and that it is serologically similar to the bacilli, made it probable in the opinion of Dienes that the L1 is a changed form of the streptobacillus and not a symbiont. His early observations on pleomorphic bacteria (9) indicated that the phenomena observed in *S. moniliformis* are not exceptional but occur in other bacteria.

A similar point of view concerning the relationship of L1 and the bacilli was taken by Dawson and Hobby (6), who studied the serological reaction of the bacilli and L1 with an agglutinin absorption technique and found no marked serological difference between them. The characteristics of L1 did not seem to Dawson (5) to exceed the morphological variations observed in bacteria. Oerskov (67) came also to a similar conclusion by direct microscopical study of agar cultures of *S. moniliformis* and L1.

The identity of L1 and the bacilli was accepted also by Heilman (41) and by Brown and Nunemaker (4). These authors studied carefully the reappearance

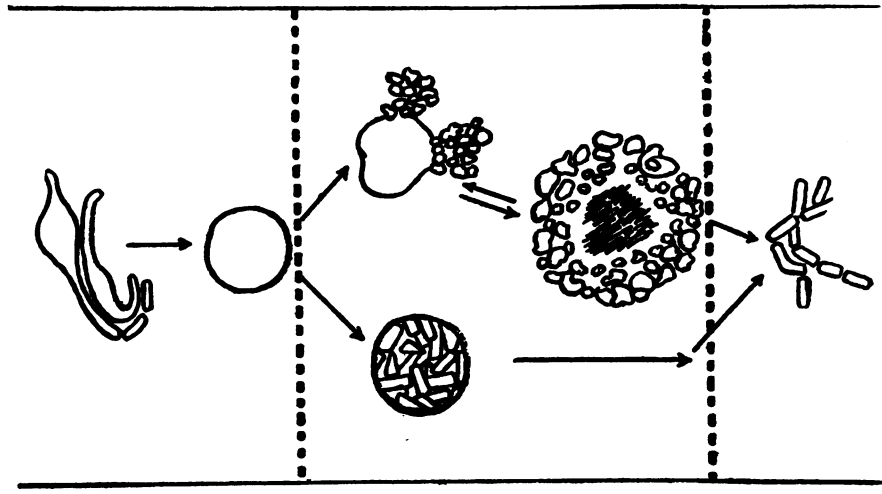
of bacilli in the cultures of L1. Heilman isolated L1 strains from seven different strains of *S. moniliformis*. They were stable on agar, but in broth, all reproduced the bacilli. With the lapse of time it required a longer period of cultivation in broth or several transfers to regain the bacilli, and after varying lengths of time, all L1 strains lost the ability to return to bacilli. In two strains it was lost after 35 and 50 transfers respectively. Brown and Nunemaker subcultured an L1 strain on solid medium 120 times without loss of its ability to return into bacillary form. Both authors made the transfers from single colonies. It is obvious from these observations that the bacillus cannot persist in the L1 colonies as a contaminant passively carried. It cannot multiply in the usual bacillary form because this grows much faster than the L1 and would be apparent in the cultures. The observations of Heilman and Brown and Nunemaker admit no other explanation but that the bacilli grow from the L forms. The reappearance of the bacilli in the L1 has not been observed directly under the microscope. The L1 never returns *en masse* into bacillary form. According to Dienes' calculations, in the best conditions only one in many millions of L1 organisms will reproduce the bacilli. The loss of the ability to return into the original form is not rare in bacterial variants.

Heilman (42) studied the growth requirements and the metabolic activities of *S. moniliformis* and L1. The bacillus grew on certain media in the absence of native animal proteins. The L1 grew only if such proteins were present. The metabolic activities of L1, especially sugar fermentations, were similar to those of the bacillus but much less intense. These observations gave further support to the essential identity of the bacillus and L1.

The conditions under which the bacillus is transformed into the L form were discussed by Dienes (13). The tendency of different strains to be pleomorphic and to produce L1 varies considerably but remains relatively constant within one strain. Strains isolated from pathological processes are usually highly pleomorphic, while the strains isolated from the pharynx of rats are often only slightly pleomorphic and produce few L1 colonies. Some strains of *S. moniliformis* tend to autolyze and to die out. Some of these strains grow in the bacillary form in broth but, when transferred to blood or serum agar, all the bacilli after a short period of multiplication swell into large bodies, and growth continues only in the L1 form. These, when transplanted to broth, reproduce the bacilli again. In all strains certain bacteriostatic agents have a similar effect. They induce all viable bacilli to swell into large bodies and growth continues only in the L form. Such observations suggest that the transformation of the cultures from the bacillary to the L form depends on an environment unfavorable to the bacilli and less unfavorable for the L1. The effect of penicillin (71) is most striking. The bacillus is very sensitive to penicillin; the growth of some strains is inhibited by the presence of 0.02 unit per ml of the medium. L1 grows even in the presence of 10,000 units of penicillin per ml, and strains of L1 occurring spontaneously in a culture are just as resistant as those developing in the presence of penicillin. L1 also developed from the bacillus on agar containing appropriate concentrations of glycine and carboxylmethoxylamine (33).

The agar seems to be important for the growth of L1. The pleomorphism of *S. moniliformis* is less apparent in broth than on agar, and the development of L1 from bacilli has not been observed in broth. L1 transferred to broth usually produces few, large compact colonies adhering to the wall of the culture tube. Growth is much better in Brewer's medium. On coagulated egg, development is very slight, few microscopical colonies develop, consisting of one layer of organisms, and the medium is not invaded.

The morphological transformations observed in cultures of *S. moniliformis* are represented in a diagram in figure 1. The consecutive steps of their transforma-



The bacilli develop into large bodies by gradual swelling.

The large bodies either develop into L forms or reproduce the bacilli within themselves.

The bacilli are regained from the L forms or directly from the large bodies produced by the bacilli.

FIG. 1. Transformation processes observed in *Streptobacillus moniliformis.*

tion were reconstructed from various observations, especially from stained agar preparations made at short intervals from growing cultures. These processes were observed later in slide cultures from single cells in *Bacteroides.*

Bacteroides. The second group of bacteria, the L forms of which were thoroughly studied, were gram negative anaerobic bacilli belonging to the genus *Bacteroides.* The L forms were studied by Dienes (12, 23, 32), Smith (80), Klieneberger (51) and by Smith, Hillier and Mudd (82). All these authors agree that these L forms are similar in the appearance of the colonies and in cellular morphology to L1. In the one strain of *Bacteroides* (strain 132) the derivation of the L forms from the bacilli was clearly apparent as were also the conditions under which that process occurs. This strain was used in most of the studies made on Bacteroides.

Both the bacillary and the L forms of *Bacteroides* grow only under anaerobic

conditions and the L forms only in the presence of animal serum. The L forms of *Bacteroides*, like the bacilli, produce gas, though much more slowly, in Brewer's medium. The cultures of the L forms and of the bacilli have the same characteristic odor, indicating a similarity of their metabolic processes. The serological properties of the L forms have not been studied.

The cultures of strain 132 on agar consisted of regularly shaped small bacilli for a period following isolation. Growth started in similar form in liquid media, but after three hours of incubation, the bacilli developed into short filaments which later showed a kink and a swelling at their center. The swelling increased in size and after 12 to 17 hours of incubation, the culture consisted of large bodies more like yeasts than bacilli. If the broth culture was transferred to fresh medium, either agar or broth, at any time during the development of the large bodies, the filaments including the swellings segmented within a short time into small bacillary forms, which continued to multiply in such forms. The bacilli are preformed in the swellings as well as in the filaments. In 24-hour or somewhat older broth cultures, almost all large bodies were viable, but when transferred from broth to agar, they behaved in a different way. Some developed into bacilli, but segmentation occurred only after a considerable growth and deformation of the large body. The bacilli are more thoroughly changed in the older than in the younger large bodies and regain their usual forms less easily. Other large bodies of 24-hour broth cultures transferred to agar develop into L forms. This process occurs in the same way as in *S. moniliformis*. The large bodies produce L forms only on agar. With increasing age of the culture, viability of the large bodies decreases, and the proportion of large bodies developing into L forms increases. The development of the large bodies into L forms and into bacilli was observed in slide cultures and photographs were published of consecutive stages of these processes (32).

The phenomena just described could be observed only for a short period following the isolation of the strain. After a few transfers either to agar or broth, the pleomorphism disappeared, growth in broth became more abundant, and no L forms were produced. Pleomorphism persisted only in cultures preserved by refrigeration in a CO_2 icebox. Pleomorphism could be restored, however, by passing the strain with the help of penicillin through the L form (23). The cultures regained from the L forms were as pleomorphic as after the original isolation and again lost their pleomorphism after a few transfers.

The observation that the pleomorphic forms of young broth cultures segment immediately into bacilli when they are transferred to fresh medium suggests that the pleomorphism is produced by the metabolic products accumulating in the medium. This conclusion is supported by the observation (23) that addition of penicillin to the broth induces the swelling of bacteria into large bodies in an otherwise non-pleomorphic strain. The large bodies so produced after a short exposure to penicillin developed into bacilli; after longer exposure to penicillin (2 days), L type colonies were produced exclusively. The effect of penicillin is apparently similar to that of the metabolic products. L colonies develop in pure culture if strain 132 is inoculated on serum agar plates containing penicillin. Such L type cultures, once isolated, grow equally well with or without penicillin.

The L type cultures are stable on agar. The L forms cannot be induced to grow in horse serum or ascitic fluid broth but they produce large fluffy colonies in Brewer's thioglycolate broth with ascitic fluid added. This medium contains 0.2% agar (23). The bacilli recur in these cultures if they are incubated for long periods. In one experiment Dienes passed two L cultures, isolated from strain 132 and another *Bacteroides* strain, repeatedly on agar plates with high penicillin concentrations, then in 15 transfers on penicillin-free agar, always making the transfers with a few colonies. From time to time the cultures were transferred to broth. The bacilli reappeared in all broth cultures within from 4 to 32 days' incubation. It is unlikely, as in the case of L1, that the bacilli were carried passively or multiplied in the L colonies during this period because they grow much faster than the L forms. The bacilli regained from the L form were similar in every respect except pleomorphism to the original bacillary cultures.

Only a few *Bacteroides* strains, usually those isolated from pathologic processes, are pleomorphic and produce L forms spontaneously. L type colonies develop more often under the influence of penicillin but not as regularly as in *S. moniliformis*. The irregularity of the results may be due partly to the inadequacy of the medium. It is also possible that the genus Bacteroides, as it is defined at present, is a heterogeneous group.

The most important results of the study of Bacteroides are the clear observation of the consecutive steps of the transformation from bacilli into L forms, the recognition of the connection between metabolic products and pleomorphism of the culture, and the observation of the reappearance of the bacilli in the broth cultures of the L forms. The observations support the genetic identity of the L forms and of the bacilli in Bacteroides as well as in *S. moniliformis*.

Proteus. The L forms of *Proteus* were studied by Dienes (18, 25), by Tulasne (85, 87) and by Freundt (37).

A few large bodies are often seen in young agar cultures of *Proteus* among the spreading filaments. Slight injuries to the filaments such as transfer to tap water or to ascitic fluid or exposure to low temperature greatly increase the incidence of large bodies. The spreaders of different strains are often antagonistic to each other, and they are transformed into large bodies at the zone of contact when the spreading cultures come together on the surface of the agar. None of these influences has any effect on the small bacillary forms.

The large bodies produced from the filaments are viable. They either return to bacilli or produce tiny L type colonies. Both processes occur in a similar manner as was described with Bacteroides. An attempt to isolate the tiny L colonies with a micromanipulator was not successful (19). They could be isolated and grown in pure culture only after much experimentation with the help of penicillin.

Proteus strains cultivated from urine and stool specimens are usually greatly inhibited by 10 units of penicillin per ml of the medium and growth is usually absent with 50 units. When exposed to penicillin in broth, all viable bacilli swell into large bodies which remain alive for several days but show no reproductive processes in the broth. Transferred to agar they produce either bacilli or L forms. On agar plates the number of colonies decreases gradually with increasing

concentration of penicillin. The bacilli become more and more pleomorphic. They grow in long filaments which develop fusiform swellings and later are transformed into large bodies. The pleomorphism of these colonies is similar to that of *S. moniliformis*. These colonies are bacillary colonies produced by the growth of the usual bacilli. There is no gradual transition between the bacillary and L type colonies.

The bacilli do not multiply on agar plates containing higher concentrations of penicillin (several hundred to several thousand units). Within a few hours the viable bacilli, the small bacilli as well as the filaments, swell into large bodies, and somewhat later, small granules grow into the agar from many large bodies. On most media, the granules do not develop beyond this initial stage and later disappear. When the medium is appropriate, the granules develop into tiny L colonies, which after two or three days, become macroscopically visible. In most *Proteus* strains, a few colonies develop to a much larger size. After five to seven days' incubation, the plate is densely covered by many tiny colonies and a moderate number of larger colonies (1 to 2 mm). Dienes (25) designated the small colonies as 3A, the large colonies as 3B. The structure and morphology of both are essentially similar and correspond to the L type colonies in other species. The growth requirements and the potential development of these two types of colonies are different. The small 3A type develops only in soft agar and only in the presence of animal serum. The type of serum exerts great influence on their development. They can be kept growing without difficulty in subcultures on appropriate media. The colonies begin to develop in a similar way, whether or not penicillin is present, but in the absence of penicillin, if the cultures are kept for a long period, the bacilli reappear and overgrow the plates. This usually does not occur in less than 24 hours, and the cultures can be propagated in the L form by daily transfers on penicillin-free agar. The reappearance of bacteria becomes increasingly delayed and may require several weeks, if the L strains are cultivated several months in the presence of penicillin. In penicillin-containing plates, the L colonies continue to increase in size for several weeks, and slowly an abundant, opaque, hard, confluent culture is produced which adheres firmly to the medium. The appearance of such old cultures differs considerably from the L1. However, the similarity in the morphology of the individual organisms remains unchanged, and the young colonies growing in fresh transplants are similar to L1.

The large 3B colonies can be induced only with difficulty to grow on the surface of the medium. Such colonies develop abundantly in pour plates made with nutrient agar containing several hundred to several thousand units of penicillin and inoculated heavily with *Proteus* bacilli (28). Horse serum favors their growth but is not necessary for it. They grow equally well in hard and in soft agar. Under appropriate conditions about 1 of 20 viable bacilli develops to 3B colonies. These colonies transferred to penicillin-free media reproduce bacilli within a few hours. This occurs also after several months of cultivation in the presence of penicillin. The bacilli start to grow from the large bodies in the same way as was observed in Bacteroides. However, some of the large

bodies develop to tiny L type colonies. L type colonies [3A] develop abundantly if the 3B colonies are inoculated on the surface of soft horse serum plates containing penicillin. The multiplication of the small forms in the 3B colonies could not be observed on the surface of agar.

The 3B colonies are not formed by penicillin-resistant bacilli. Their morphology is similar to the 3A colonies and differs considerably from the morphology of bacillary colonies changed by penicillin. The bacilli recovered from 3B colonies are not resistant to penicillin, even if these colonies are grown for several months with a high concentration of penicillin. The growth of 3B colonies is not made possible by a permanent change in their metabolism, but is connected with a change in morphology and disappears when the usual bacterial morphology is resumed. The 3B colonies grow equally well with any bacteriostatic concentrations of penicillin; they are not penicillin-dependent. They have no increased resistance to streptomycin or to glycine.

The serological and metabolic properties of 3A cultures were studied. Dienes, Weinberger and Madoff (34) produced agglutinating sera in rabbits with three *Proteus* strains and the corresponding L cultures. The antigen specificity of the different strains was markedly different and the bacillary and L forms of two strains reacted similarly both *in vivo* in the production of antibodies and *in vitro* in the agglutination test. The bacilli and the L forms of the third strain reacted only slightly with each other's sera. The serum made with the L form of this strain reacted with other *Proteus* strains to a certain degree and in this respect was a Proteus antiserum. No overlapping was present between the sera made with *Proteus* and *Salmonella* L strains.

In the agglutinin absorption test, the bacilli absorbed the agglutinins both from the bacillary and L sera of one strain, while the L culture did not absorb the agglutinins from the bacillary sera. Apparently not all of the bacillary antigens are present in sufficient amount in the L cultures. Tulasne (87) observed the absence of H antigen in the L form, while the other antigens of the bacillus were present in it.

The *Proteus* L type cultures grew only on agar. They did not grow on other solid media such as coagulated egg or serum. Growth was not obtained in broth or in Brewer's medium. Occasionally growth occurred in serum broth when a small piece of sterile cotton or cellophane was rubbed over the agar colonies and submerged in the medium (28). Apparently, the physical properties of the medium exert as great an influence on the growth of 3A colonies as the presence or absence of certain nutrients.

The 3A cultures present one of the most characteristic metabolic properties of Proteus, the fermentation of urea. A slight digestive effect is apparent in sedimented boiled blood agar plates. Coagulated serum incorporated in agar is not digested. Fermentation of sugars has not been studied.

All *Proteus* strains freshly isolated from urine and stool specimens produce abundant 3A colonies. Considerable difference between strains is apparent in the *viability* of 3A colonies *and* in the *occurrence* of 3B colonies, on the surface of the agar. Freundt (37) examined 31 *Proteus* strains, partly freshly isolated,

partly stock strains. All produced L type colonies with penicillin but some strains only in the presence of certain concentrations of penicillin. The behavior of *Proteus* X2 and X19 differs from other *Proteus* strains. They are very resistant to penicillin, and although L type colonies developed on penicillin plates, it was not possible to keep them growing in subcultures. The L type colonies produced by an XK strain grew well in subcultures. In addition to the X2 and X19 strains carried in the laboratory, which were probably derived from the strains isolated by Weil and Felix, the reviewers (28) studied six X strains, recently isolated by Kauffmann and Perch (43) in Denmark.

Freundt (37) and Tulasne (87) did not distinguish between the 3A and the 3B colonies and for this reason it is difficult to compare their observations in detail with those of the reviewers. Freundt observed the development of large bodies and L forms in slide cultures and his drawings give a clear impression of these processes. He observed that in some colonies the L forms returned into bacillary forms which during the further development of the colony again resumed the morphology of L forms. The description of the morphology of L colonies given by these authors agrees well with that of the reviewers. Tulasne (88) described recently growth of L forms (probably 3B) as a film on the surface of broth. A filtrate obtained from this film through a porcelain filter (filtré sur bougie) reproduced L colonies in the presence of penicillin and bacterial colonies in its absence.

The diagram in figure 2 gives a brief survey of the transformation processes observed in Proteus. All the different transformations except the growth of bacilli from the 3A colonies were observed directly under the microscope.

The most important observation made with Proteus is the ease and regularity with which the bacilli reappear in L cultures. If doubt exists as to the identity of *S. moniliformis* and L1, this hardly can be maintained in Proteus. The metabolic and serological properties of the L strains and of the bacilli also support the conclusion that they are forms of the same organism.

Salmonella. Observations on the L forms of *Salmonella* were published only by Dienes and his associates (22, 23, 93). Freundt (37) mentions that he observed development of L type colonies in Salmonella.

Pleomorphic strains are rare in Salmonella and spontaneous development of L forms has not been observed. The reviewers studied seven freshly isolated strains of *S. typhosa*, one of *S. enteritidis* and five of *S. typhimurium*. Several *S. typhimurium* and the *S. enteritidis* strains when transferred to soft horse serum plates containing a few hundred to several thousand units of penicillin per ml reacted in the same way as Proteus. Three A and 3B colonies with similar properties grew on the plates. Three B colonies are not as easily produced in Salmonella and could be propagated in subcultures only in pour plates. The bacilli are reproduced from the 3B colonies in the same manner as in Proteus. Three B L type colonies have not yet been obtained from *S. typhosa*. The great variability of their production in one species, *S. typhimurium*, suggests that their production probably depends to a large extent on environmental factors.

The small 3A colonies grew with or without penicillin but only in the presence

of animal serum on soft agar plates anaerobically incubated. The source of the serum was of importance. The colonies grew best with horse serum. Rabbit serum was less favorable and no growth was obtained with mouse or guinea pig serum. The L strains isolated with horse serum could be adapted only with

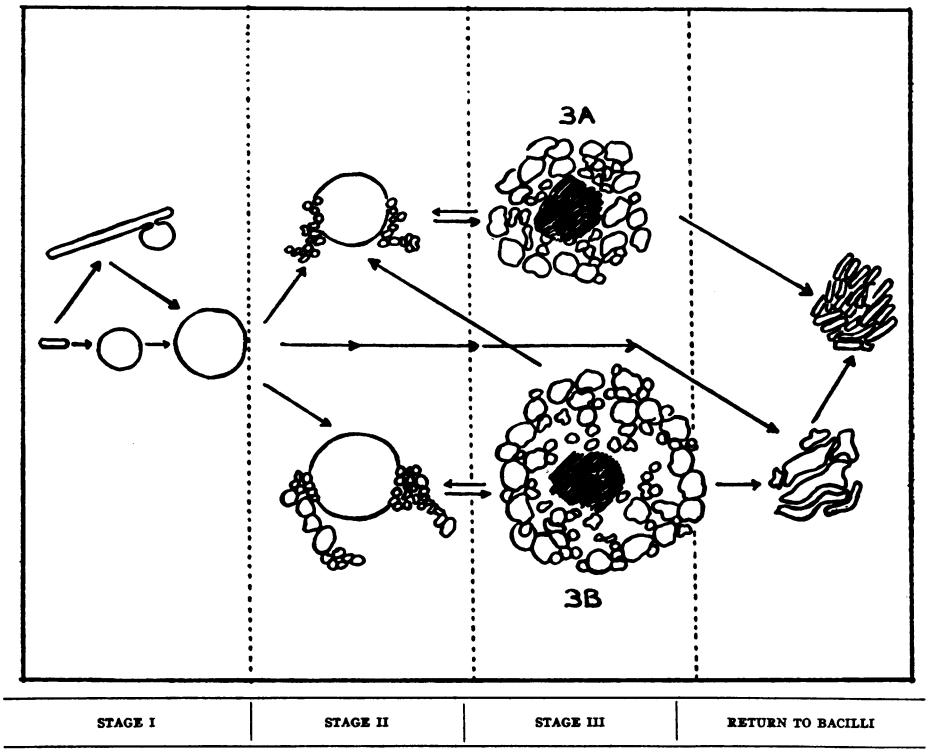


FIG. 2. Transformation processes observed in Proteus cultures.

difficulty to grow with rabbit serum. They did not grow in broth but grew in Brewer's medium. The young 3A type colonies are similar in appearance to L1. On appropriate media, they may grow to a larger size (2 mm) as moist transparent colonies with an opalescent center embedded in the medium. Growth in occasional cultures progresses for several weeks, and large opalescent colonies develop comparable in abundance to bacterial colonies and to old colonies of *Proteus* L. The morphology of these colonies remains the same as that of the

usual small colonies. The bacilli have not been recovered either in agar or in broth from the 3A cultures.

With *S. typhosa* and *S. typhimurium*, several antibiotic and bacteriostatic chemicals were tested for ability to induce transformation into L forms (33). Aureomycin and chloramphenicol induced, in concentrations just inhibiting bacterial growth, the swelling of a few bacilli to large bodies and from some of these, tiny L growths started. The development of L colonies was very limited, probably because the inhibitive concentration of the antibiotic for bacteria and for the L type cultures is about the same. With streptomycin and terramycin, no L forms were observed. From among many chemicals tested, *dl*-methionine (28), *l*-phenylalanine (28), glycine and carboxylmethoxylamine induced the growth of L type colonies, but only in a narrow concentration exceeding the inhibitory dose for the bacilli. The bacilli swelled into large bodies and L colonies grew from them as with penicillin. All the other bacteriostatic chemicals tested were at least equally toxic to the L forms as to the bacilli.

It is of great interest that *S. typhosa* could be transformed into L forms by exposure to antibody and complement. Rabbit antibody with guinea pig complement and fresh convalescent serum of patients with typhoid were especially effective. The bacilli after 15 minutes or more exposure were transferred to soft horse serum agar. A moderate number of the bacilli swelled on the agar into large bodies, some of which developed typical L type colonies. In some plates among a few bacillary colonies several hundred L type colonies developed. The bacilli exposed to normal human and rabbit serum also occasionally produced a few L type colonies. The morphology, serological reactions and penicillin resistance of these L colonies obtained by serological reaction were similar to those isolated by penicillin. *S. typhimurium* was not lysed by antibody and complement and produced no L type colonies when it was exposed to them.

L type colonies were observed also in typhoid cultures exposed to bacteriophage. They did not develop from the lysed bacilli but from tiny secondary colonies which developed on the lysed areas of agar plates. The bacilli swelled to large bodies in some of these, and L type colonies started to grow from these large bodies.

The serological reactions and the sugar fermentation of a *S. typhosa* strain, or a strain of *S. typhimurium* and of the L forms isolated from them were studied (93). The sera produced in rabbits with the bacilli and the L form reacted equally well with both. The overlapping in the serological reactions between the two species was about the same with the L form as with the bacilli. No overlapping was present between the L forms of *Salmonella* and *Proteus*. The sugar fermentations of the L forms were less intensive than those of the bacilli but otherwise they were similar. The *S. typhimurium* L produced acid and gas with glucose and acid with maltose. Lactose and sucrose were not fermented. The L form of typhoid bacilli produced acid and no gas in glucose and did not ferment lactose. The tests with maltose and sucrose did not give clear-cut results.

The considerations which apply to the relationship between the bacilli and

the 3B L type growth in Proteus are applicable also to Salmonella. The return of the 3A cultures to bacilli has not been observed in Salmonella, but the similarity of their serological and metabolic properties to those of the bacilli indicate that they are a growth form of the bacilli. The production of L forms by serological reactions and by bacteriophage is of great interest because this indicates that L forms are produced under certain conditions which may occur in the natural environment of the bacilli.

Shigella. Development of L type colonies was not observed with old laboratory strains of *Shigella* (93). They developed in a few freshly isolated Flexner strains when they were studied with a technique similar to that used with Salmonella. The L cultures could be kept growing in subculture only with difficulty and never more than a few colonies developed on the plates. *Shigella sonnei* was very resistant to penicillin. A few bacillary colonies developed with as high concentration as 1,600 units of penicillin per ml. Large bodies were produced abundantly from the bacilli on the surface of the agar and from many of these L type colonies started to grow. They failed to grow to a larger size and could not be propagated in subcultures.

Escherichia coli. Occasional strains of *E. coli* isolated from infected urines are spontaneously pleomorphic. Dienes (9) observed in 1939 that the large bodies of some pleomorphic strains grow in transplants into tiny L type colonies. On one occasion the reproduction of bacilli inside the large bodies was observed (10).

Two pleomorphic colon bacillus strains were more thoroughly studied (13). In the cultures of one strain, 1706, masses of L granules grew into the agar under the colonies preceding the autolysis of the large bodies. The large bodies of the other strain produced L granules only when they were transferred to fresh medium. Growth of the L forms in subcultures could not be induced.

E. coli is more resistant to penicillin than Salmonella, and regardless of the concentration of penicillin employed, only a small proportion of the bacteria swell into large bodies. Small L type colonies develop on penicillin plates, as in naturally pleomorphic strains, but they remain very small and thus far have not been grown in subcultures.

Vibrio comma. Minck reported the development of L type colonies on soft serum agar plates containing penicillin. Subcultures were not successful (62).

Hemophilus influenzae and Hemophilus parainfluenzae. Type B strains of *H. influenzae* isolated from blood cultures are usually highly pleomorphic. Such strains are found less often in spinal fluid and are rarely isolated from the pharynx or sputum. The pleomorphism is not clearly visible in smears, but it is apparent in wet stained agar preparations. The appearance of the colonies sometimes is similar to that of *S. moniliformis.* In certain strains, as in the colon bacillus 1706, preceding autolysis, many tiny L type colonies grow into the medium from the large bodies. These tiny colonies could not be cultivated in transplants.

Transferred to plates containing penicillin, all viable influenza bacilli swell into large bodies, and after a few days varying numbers of fairly large L type colonies develop (28). These colonies are similar in structure and morphology to the large 3B L type colonies of *Proteus* and *Salmonella.* Growth of these colonies

was obtained only in one consecutive transplant on plates containing penicillin (28). Upon transfer to penicillin-free media, the usual colonies of *H. influenzae* develop. Similar 3B L type colonies develop on plates containing appropriate concentrations of glycine and methionine (28). The growth of these colonies is not dependent upon the physical consistency of the medium, the source of animal protein, the concentration of penicillin or anaerobic incubation. The bacilli are reproduced from the large bodies of *H. influenzae* in the same manner as in *S. moniliformis* inside of the large bodies.

Colonies corresponding to the 3A L type (of Proteus) are usually not observed on penicillin plates. Dienes (28) reported the isolation of an L strain, corresponding to the 3A type growing without difficulty in subcultures, from a pharyngeal culture of *H. influenzae*. The relationship between the L forms and the bacillary cultures could not be confirmed serologically because the bacillus was lost. This single successful isolation of 3A L type colonies of *H. influenzae* stands against many negative attempts. Since pleuropneumonia-like organisms often can be isolated from throat cultures, contamination of the influenza strain with such organisms in this one isolation cannot be excluded. Strains of *H. parainfluenzae* and *H. hemolyticus* are often pleomorphic and produce spontaneously tiny L type colonies (15). On penicillin plates sometimes 3B L type colonies develop (28).

Pasteurella. Two strains of *Pasteurella* isolated from the nasal cavities of rabbits were studied on penicillin plates (28) by the reviewers. The bacilli swelled into large bodies but L type colonies were not observed. Tulasne (85) mentioned without detail the occurrence of L forms in *P. pestis*. Klieneberger (52) also described L forms in this species, but in the opinion of the reviewers, it is probable that the forms observed were pleomorphic bacilli.

Neisseria. N. gonorrhoeae cultivated directly from patients are often pleomorphic, and it is apparent in wet stained agar preparations that the cocci swell into large bodies similar to those of gram negative bacilli. Small pleomorphic colonies of *N. gonorrhoeae* easily can be mistaken for pleuropneumonia-like organisms in impression preparations. Tiny L type colonies grow under some of the pleomorphic coccal colonies (11). These L type colonies are very small and soon disappear. Their nature would not be recognizable without reference to other species in which the L type colonies are larger and grow in subcultures. All viable cocci swell into large bodies on penicillin plates, and the development of tiny L type colonies is rarely seen. They disappear early and do not develop in transplants.

Brown and Hayes (3) reported the isolation and subculture of L type colonies of *N. gonorrhoeae* on plates containing sulfonamides. The reviewers were unable to confirm these observations. Morton *et al.* (63) reported recently that pleuropneumonia-like organisms are often able to grow under a bacterial colony on media otherwise inappropriate for their growth. Perhaps the L type colonies observed by Brown were pleuropneumonia-like colonies growing in association with the cocci.

Spontaneous pleomorphism occurs rarely in *N. meningitidis*. On penicillin plates, the cocci swell into large bodies. L forms develop from *N. meningitidis*

more often than from *N. gonorrhoeae*. It was not possible to grow these tiny colonies to a larger size or to propagate them in transplants (28).

Miller and Bohnhoff (61) reported that streptomycin-resistant and dependent *N. meningitidis* colonies develop on plates containing high concentrations of the antibiotic. The properties of these colonies are not suggestive of L type colonies. Streptomycin did not induce swelling of the cocci, or transformation into L forms in experiments carried out by the reviewers.

N. catarrhalis strains isolated from the pharynx swell on penicillin plates into large bodies, and in some strains tiny L colonies develop abundantly from the large bodies (28). Thus far these L forms have not been propagated in subcultures.

Gram positive bacilli. L type colonies were isolated from five strains of large gram positive bacilli by Dienes (26, 27). Only one of these, a strain of *Clostridium tetani*, was definitely identified. Another strain was identified as *Clostridium* but the species was not determined. Two of the other strains grew equally well aerobically and anaerobically and should be classified in the genus *Bacillus*. The classification of the fifth strain is uncertain.

C. tetani did not grow and did not change in morphology on serum agar containing 400 and 1,600 units of penicillin per ml. Soft horse-serum agar plates without penicillin were inoculated from a broth culture and about 1,000 units of penicillin were deposited in small troughs made in the agar (27). Three zones were present on these plates after two days' anaerobic incubation. No growth developed in the area immediately surrounding the penicillin trough. Adjoining this area was a zone of bacterial colonies of increasing size which were lysed by the diffusing penicillin. Beyond this area, the bacilli grew as a spreader over the plates. In the lysed colonies, the bacilli were swollen into large bodies. These were very large (10 to 20 μ) and many L type colonies developed from them. They grew without difficulty in subcultures. The properties of these cultures thus far have not been studied in detail. The morphology as seen with low and high magnifications is similar to that of L type colonies isolated from gram negative bacilli. Animal serum is not necessary for their growth. No growth was obtained in liquid media and the bacilli could not be regained from the L type cultures. The latter injected into mice did not produce tetanus.

The second Clostridium was obtained from a lung at postmortem examination. No sign of gas bacillus infection was present. L forms were not obtained from it on penicillin plates or by using the technique successful with *C. tetani*. When the colonies in a two-day-old agar culture were streaked out on the uninoculated areas of the same plate, a wide zone of inhibition was noted around the original growth. The bacilli in the area of inhibition swelled into large bodies some of which developed into L type colonies (26). The L type colonies were similar to those of *C. tetani* and they grew better in the absence of horse serum. The presence of penicillin exerted no effect on their growth.

L type colonies were isolated with the same technique as used with tetanus from a large aerobic gram positive bacillus probably belonging to the genus *Bacillus* (26). It was designated as *Bacillus 3*. This bacillus produced spores

abundantly and L forms were isolated from the bacilli obtained from heated spores. The L colonies obtained were similar to those of *C. tetani*. L type colonies were isolated from two more gram positive aerobic bacilli. One (*Bacillus 1*) was isolated from a contaminated blood culture, the other (*Bacillus 2*) from soil. These L cultures differed both macroscopically and microscopically from the L cultures of *Clostridium* and *Bacillus 3*. The colonies had only a small opaque center grown into the agar and a very fine circular periphery on the surface. The colonies consisted of very small bacillary granules which swelled only moderately on the surface of old colonies to the size of 2 to 3 microns. Their morphology is quite similar to that of the filterable organism isolated by Laidlaw and Elford (58) from sewage. However, the L type cultures could not be induced to grow in broth in which Laidlaw and Elford's organism grew abundantly. The appearance of these cultures on serum agar is very characteristic. After the original small colony is formed, the organisms extend in small streams both on the surface and into the agar and within a few weeks produce many small secondary colonies. An area of several square millimeters can be covered in this manner. The L cultures isolated from aerobic large gram positive bacilli grew better in the absence of animal serum. Some grew only on hard agar, some only on soft agar. In the case of the two last-mentioned bacilli, starch markedly increased their growth. Broth cultures were not successful and the bacilli were not recovered from the L forms.

In a strain of *C. perfringens* exposed to penicillin, the development of large bodies and tiny L type colonies was observed by Dienes (26). A strain of *B. anthracis* isolated in his laboratory did not produce large bodies or L type colonies in the presence of penicillin (28). Many other aerobic and anaerobic gram positive spore-bearing bacilli isolated from contaminated media and from soil were studied on penicillin plates. None, with the exception of the strains described, produced L type colonies.

Bacilli of uncertain classification. Klieneberger (49) isolated an L type culture from a bacillus obtained from suppurating lymph nodes of guinea pigs. The bacillus resembled *S. moniliformis* in many respects. The cultures were lost during the war and were not studied further.

After the discovery of the L1, the next L type culture observed was obtained by Dienes from a bacillus cultivated from a human dog-bite wound (9, 13). The bacillary cultures were distinctly yellow and the most probable classification was as a Flavobacterium. The development of agar cultures immediately after isolation of the strain was unusual. The small bacilli, transferred to agar plates started to multiply and within a few hours produced tiny colonies. Then multiplication stopped and the bacilli became pleomorphic and swelled into large bodies. Where the inoculation was heavy the small bacilli reappeared and produced a heavy confluent growth, consisting of small, regular-shaped bacilli. The bacilli did not reappear where the tiny pleomorphic colonies were well distanced and these developed into L colonies. This type of growth persisted for a few weeks. Later the bacilli no longer became pleomorphic in the young cultures and did not produce L type colonies. Nine years after the original isolation of the strains, L forms were again produced on penicillin plates. The

L cultures were similar in appearance to the 3A L cultures of *Proteus*. They did not grow in broth and the bacilli could not be regained from them.

TABLE 1A

Summary of observations on L type cultures in bacterial species

(Species most thoroughly studied)

BACTERIA EXHIBITING L TRANSFORMATION	OCCURRENCE OF L TYPE CULTURES	TYPE OF L COLONIES	CULTURAL REQUIREMENTS	REVERSION TO BACILLI
Streptobacillus moniliformis (4, 8, 41, 46)	Spontaneous, or by penicillin, glycine or carboxylmethoxylamine	3A	Serum agar	In broth
Bacteroides (23, 32, 51)	Spontaneous, or by penicillin	3A	Serum agar, anaerobic	In broth
Proteus (25, 37, 87)	By refrigeration, transfer into tap water, strain antagonism, penicillin, or carboxylmethoxylamine	3A	Soft serum agar	In broth and on agar
	By penicillin	3B	Agar (serum not required)	In broth and on agar (immediately on penicillin-free media)
Salmonella typhosa (33, 93)	By penicillin, glycine, *dl*-methionine, *l*-phenylalanine, aureomycin, carboxylmethoxylamine, chloramphenicol, antibody and complement, or by bacteriophage	3A	Soft serum agar; anaerobiosis favorable	Not observed
Salmonella typhimurium, S. enteritidis (33, 93)	By penicillin, glycine, *dl*-methionine, *l*-phenylalanine, aureomycin, carboxylmethoxylamine, or by chloramphenicol	3A	Soft serum agar, anaerobic	Not observed
	By penicillin	3B	Agar (serum not required)	In broth and on agar (immediately on penicillin-free media)

An L culture developed on a penicillin plate from a small gram negative bacillus (isolated (28) from the trachea of a dead guinea pig) whose appearance and

cultural properties conformed to Pasteurella. The L type cultures could be maintained in subcultures without difficulty.

TABLE 1B

Summary of observations on L type cultures in bacterial species

(Species in which L forms were isolated but were not thoroughly studied)

BACTERIA EXHIBITING L TRANSFORMATION	OCCURRENCE OF L TYPE CULTURES	TYPE OF L COLONIES	CULTURAL REQUIREMENTS	REVERSION TO BACILLI
Shigella paradysenteriae (93)	By penicillin	3A	Soft serum agar, anaerobic	Not observed
Hemophilus influenzae (21)	Spontaneous, or by penicillin or glycine	3A 3B	Blood agar Serum agar	Not observed On blood agar
Flavobacterium (9, 28)	Spontaneous, or by penicillin	3A	Serum agar	Not observed
Gram-negative bacillus from guinea pig pharynx (probably *Pasteurella*) (28)	By penicillin	3A	Serum agar	Not observed
Clostridium tetani and 1 strain unidentified (27)	By penicillin	3A	Nutrient agar (serum not required) anaerobic	Not observed
Bacillus 3 strains unidentified (26)	By penicillin	3A	Nutrient agar (serum not required) anaerobic	Not observed

TABLE 1C

Summary of observations on L type cultures in bacterial species

(Species in which the initial development of L type cultures from the bacteria was observed, but the L forms were not propagated in subcultures)

BACTERIA EXHIBITING L TRANSFORMATION	OCCURRENCE OF L TYPE CULTURES	TYPE OF L COLONIES	CULTURAL REQUIREMENTS	REVERSION TO BACILLI
Shigella sonnei (93)	By penicillin	3A	Soft serum agar anaerobic	Not observed
Escherichia coli (13)	Spontaneous, or by penicillin	3A	Serum agar	Not observed
Vibrio comma (62)	Penicillin	?	Serum agar	Not observed
Hemophilus parainfluenzae (15, 28)	Spontaneous, or by penicillin	3A 3B	Blood agar Serum agar	Not observed Not observed
Neisseria gonorrhoeae (11), *N. intracellularis* (28)	Spontaneous, or by penicillin	3A	Serum agar	Not observed

The behavior of many other bacteria on penicillin plates was studied in addition to those already discussed (28). Several strains of *Pseudomonas aeruginosa* and *Serratia marcescens* were very resistant to penicillin and their growth and

morphology were unchanged on penicillin plates. Streptococci, staphylococci, and diphtheroids were sensitive to penicillin but their morphology was unchanged by it. Some strains of alpha hemolytic streptococci spontaneously developed large bodies but further development of these was not observed.

REVIEW OF THE PROPERTIES OF L TYPE CULTURES AND CONSIDERATIONS PERTAINING TO THEIR NATURE

A brief survey of the observations relating to the L forms is presented in table 1. The information available on the different bacteria included in the table varies considerably. The first five entries in table 1A were extensively studied and the derivation of the L forms from the bacteria seems to be well established in them. The L forms were isolated in some other species but were not further studied (table 1B). In *S. sonnei*, *E. coli*, the Neisseria and some other species only the development of the tiny L type colonies was seen in the bacterial cultures (table 1C). They were not isolated. It seems justifiable to put the well studied and the less well studied species in the same table because all present the most characteristic properties of L cultures. Not only are the appearance and the morphology of the tiny colonies similar, but they are derived from the bacteria in a similar manner and under similar conditions. Under unfavorable conditions, *S. moniliformis*, the Salmonella and Proteus produce only similar tiny L type colonies. These were first observed in the two last-mentioned genera and several years passed until the conditions which permitted their continuous growth were discovered accidentally. Consideration of the properties and nature of the L forms should be based on the well studied species, but it is of interest that the initial stages of their development were observed in several genera and families. Thus, this ability to transform into such forms may well be a general property of bacteria.

Transition from bacterial to L forms occurs in all species in a similar way. The bacteria swell into large bodies and the L forms grow from these. This process was observed directly in several species (*S. moniliformis*, Bacteroides, Proteus, *E. coli*, *H. influenzae* and *S. typhosa*). In the other species the swelling of the bacilli into large bodies always preceded the development of L forms. The photographs 1 to 11 in Plate I[2] illustrate this process in Proteus.

[2] The illustrations in Plates I and II are intended to convey an impression of the actual observations of the unusual morphology of the organisms and of the fitness of the microscopic technique used for their study. It would be difficult to present convincing evidence in the illustrations for the accuracy of the interpretation of the structures visible in the photographs. The evidence for the opinions of the reviewers is presented in the text and more fully in the original papers.

Many of the photographs were made from wet unstained or stained agar cultures. The latter permit one to see not only the structure of the colonies but the morphology of the organisms. However, the individual organisms cannot be photographed from such preparations because their arrangement is tridimensional. Drying of the agar compresses the colonies vertically so that the organisms are more nearly in a plane and can be better photographed. Sometimes an excellent impression of young colonies remains on the glass after lifting the dry stained agar film. Agar fixation gives a good impression of the large 3B L colonies but it is not applicable to young or to small colonies. The L forms of different species are so similar that illustrations of any one apply almost equally well to all.

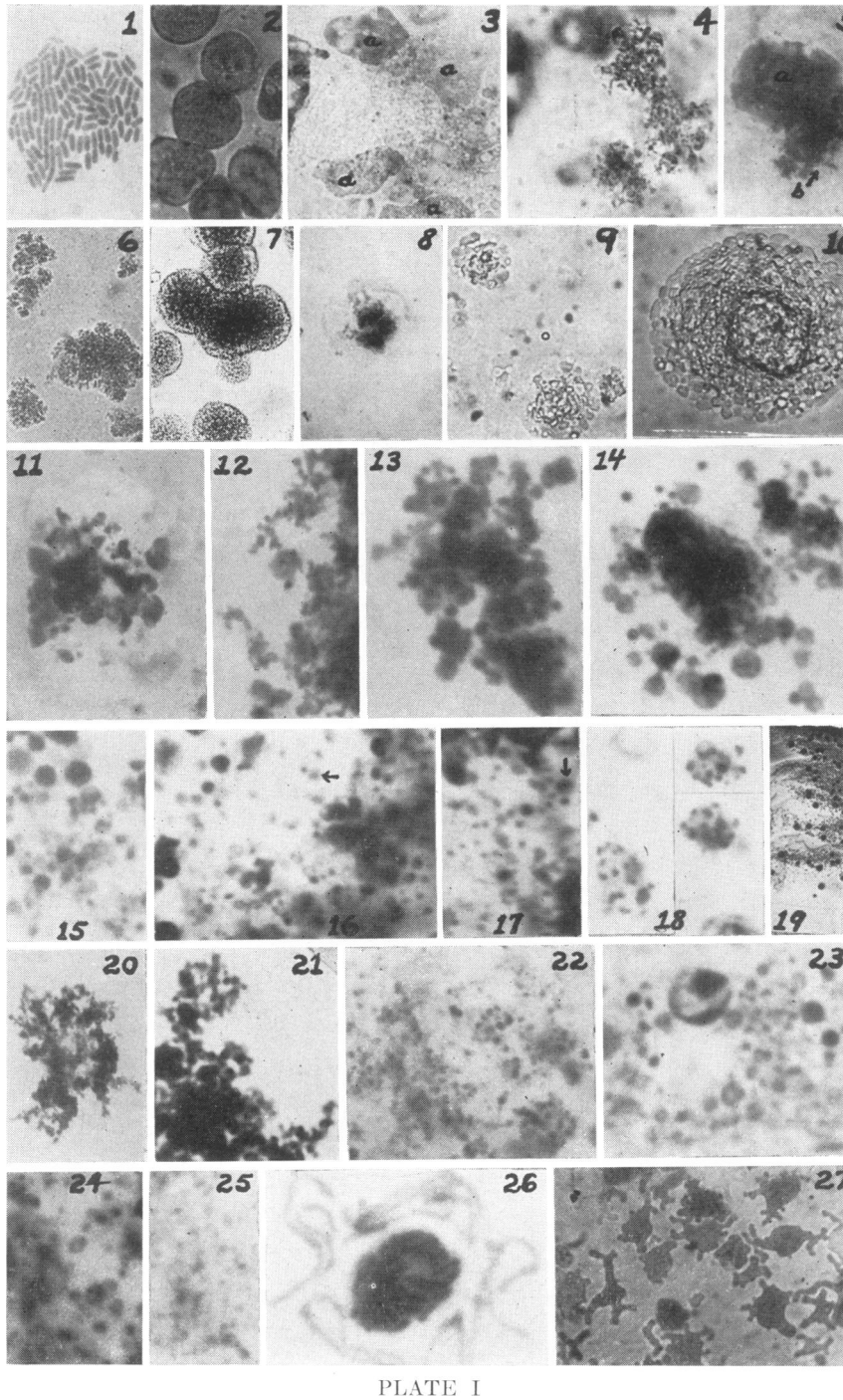

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PLATE I

The appearance, physical properties, staining reactions and autolytic phenomena of the large bodies developing from the bacilli are similar in all species. The large bodies in many species have the double potentiality of developing into L forms or of reproducing the original bacilli. It is not apparent from their morphology which course their development will take. Bacilli develop either inside the large bodies (as in *S. moniliformis* (14) (Photograph 26, Plate I),

PLATE I

Plate I illustrates the development of L colonies of Proteus, the morphology of these and similar colonies in other species and the growth of bacteria from the large bodies. Photographs 1, 2, 3, 4, 8, 11 and 27 were made from wet stained agar preparations; Nos. 6, 7, 9 and 19 were made from unstained agar; Nos. 11, 12, 13, 14, 20, 21 and 25 were made from dry stained agar preparations.

1. A young colony of Proteus (4 hours) on the surface of agar without penicillin. × 900.

2. The surface of penicillin agar inoculated at the same time as No. 1 after 6 hours' incubation. All bacilli have swollen into large bodies. × 900.

3 and 4. An area of the same plate as No. 2 after 24 hours' incubation. In No. 3 the focus is on the surface. The large bodies (marked with letter "a") are less well or not at all stained and many are vacuolated. In No. 4 the focus is lower and it can be seen that three small colonies consisting of very small organisms have started to develop under the large bodies which appear only as dark shadows. × 900.

5. A large body (a) and an L colony (b) developing from it. This photograph was made from a lightly inoculated area of the same plate as Nos. 3 and 4 from a dried stained agar preparation. × 900.

6. Young 3A colonies photographed after 2 days' incubation. × 100.

7. Well developed 3A L colonies. × 100.

8. A young 3A L colony with dark center and light periphery. This is a characteristic appearance of young L colonies in wet stained agar preparations. × 100.

9. Three small 3B L colonies after 1 day's incubation. In one, only the center of the colony is developed; in the other two the dark center is surrounded by large bodies. × 200.

10. A larger 3B colony after 2 days' incubation. Unstained agar. × 200.

11. A small 3B L colony with moderate magnification (× 900). It corresponds to the colony without peripheral large bodies in No. 8. This colony contains all the transitional forms from very small to very large. The differences between Nos. 4, 21 and 22, on the one hand, and No. 11 on the other, are characteristic of the microscopic structure of the young 3A and 3B L colonies.

12. The periphery of a 3A L colony spreading in the agar consisting of small forms. × 2,000.

13 and 14. The morphology of individual organisms in 3A L colonies from impressions obtained from dried stained agar preparations. The smallest forms are near the limit of resolution of the microscope. They are often in pairs or in short chains. The impressions represent the surface of the colony and the majority of the organisms are in the process of swelling into large bodies. × 3,000.

15, 16 and 17. The morphology of the organisms in the 3B colonies of Proteus. They were made of impressions from dried stained agar. The similarity of the organisms to those of the 3A colonies is apparent. All transitional forms are present from the smallest to the large bodies. The small forms are often in pairs and in short chains (No. 17). Two pairs are marked in which one organism has grown to a larger size than the other. It is difficult to make satisfactory photographs of the small forms because in the center of the colony they are embedded in mucoid material and are not in one plane in the preparations. × 2500.

18. Large bodies with the small forms apparent in them. The small forms are arranged

H. influenzae (28) and *E. coli* (10)), or the large bodies may first present an irregular growth and divide into segments (Photograph 27, Plate I). In this case the regular bacillary forms are restored after a few more divisions (as in Proteus (18), Bacteroides (32) and Salmonella (93)). In both cases, the large body contains many individual growing elements.

The L forms grow from the large bodies as strands of small refractile granules. In some cases the large body disintegrates into many L granules. Tulasne (88) described development of the L forms as small granules with intense Brownian movement in vacuoles which by gradual enlargement fills the whole large body. The reviewers have not observed such a process.

The manner in which the colonies develop and the appearance of a fully developed colony under low magnification are very characteristic and are seen only in cultures of the L forms and of the pleuropneumonia group of organisms. Various appearances of the young and fully developed colonies are illustrated in the plates. Dienes has never seen bacterial colonies with a similar appearance and structure during 12 years of experience with the L forms and the pleuropneumonia group.

The characteristic structure of young and mature L type colonies is largely the result of the penetration of the growing L forms into the agar. The invasion of the agar appears to be necessary for their growth. In broth, bacteria often become swollen into large bodies but further development into L forms does not occur. L type cultures isolated on agar and transferred to broth usually do not grow unless some agar (0.1 to 0.2%) is present. Occasionally, cultures adhering to cellophane or a cotton ball can be induced to grow in broth (28). On coagulated

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in pairs and short chains and vary in size like those in Nos. 16 and 17. Impression preparations from cultures stained *in situ* with methyl violet. × 2,000.

19. 3A and 3B L colonies as they appear on the agar with the hand lens. A penicillin agar plate inoculated with Proteus. × 1.5.

20. A small L1 colony. Strands of small granules spread in different directions into the agar. This appearance is characteristic of young 3A L colonies. × 900.

21. Part of an L1 colony similar to that in No. 20. Few of the individual organisms are well defined but the characteristic structure of the colony is clearly visible. × 2,000.

22. Small forms from the center of a typhoid L colony in Brewer's medium. Agar fixation preparation stained with azure II. × 2,000.

23. Edge of a Salmonella 3B colony with small forms of various sizes, probably formed by disintegration of a large body. They are similar to those in 3B colonies of Proteus. Agar fixation stained with toluidine blue. × 3,000.

24. Small forms from the center of a Salmonella 3B colony. Agar fixation stained with toluidine blue. × 3,000.

25. Small forms from the L colony of a gram positive bacillus. Impression from dry stained agar. × 3,000.

26. Large body of Streptobacillus from a few-hour-old transplant. The bacillary filaments have grown slightly and the large body is filled with bacillary forms. Agar fixation. × 3,000.

27. Large bodies of Bacteroides produced in penicillin broth transferred to penicillin-free agar starting to develop into bacteria. The large bodies are deformed; branching filaments grow out and later segment into bacilli. × 900.

serum or egg the L forms either do not grow or produce a slight growth consisting of one layer of organisms. The physical properties of the medium exert a great influence on the development of the cultures.

Although the basic structure of all L type colonies and the morphology of individual organisms is similar, the macroscopic appearance of the cultures isolated from different bacteria varies considerably and is greatly influenced by the composition of the medium and the degree of adaptation of the cultures to the medium. L type cultures immediately after isolation in most cases resemble the L1 and consist of small opaque colonies firmly attached to the agar. When the cultures become adapted to the medium, growth often continues for several weeks or months. The center of the colonies may autolyze and the growth continues at the periphery. In Salmonella and some of the gram positive bacilli an abundant moist surface growth develops. In Proteus, Flavobacterium and sometimes in Salmonella, the colonies grow to firm opaque masses adhering to the agar. In a Clostridium some of the L colonies increased slowly to a size of 10 mm and in another bacillus a creeping growth with many daughter colonies may develop (Photograph 15, Plate II).

Klieneberger (50) and Dienes agree that the only morphological elements visible in the cultures are the small forms, the large bodies and the transitional forms. It is apparent that the small forms are reproductive because they are present alone in young colonies and at the spreading edge of larger colonies. The arrangement of the small forms indicates that they multiply by division like bacteria. However, in most cases growth does not start from individual organisms, only from a heavy inoculum. The size of the smallest forms in microscopical preparations is between 0.3 and 0.5 micron. Klieneberger (53) recently measured the size of the smallest units capable of reproduction in an old L1 culture by filtration through gradocol membranes. It was 175 to 250 microns, about the same as the large viruses and the smallest reproductive forms in the pleuropneumonia group. As in the latter, only a small proportion of the organisms in the L1 cultures were of such small size. Filtration of the old L1 culture was possible because it would grow from the resultant small inoculum. In most cases filtrates, even from coarse Mandler filters, remain sterile.

Klieneberger believes that the basic form of the small granules is spherical and all elongated forms are produced by the influences of the environment. Dienes' impression is that the small forms are often elongated and distinctly bacillary. They may grow into short curved filaments and sometimes have the appearance of tiny bipolar stained bacilli (20).

The small forms gradually swell into the large forms and in most cultures the majority of the organisms are in various stages of this process. The appearance of the large bodies is entirely similar to those developing from bacteria. The impression of both Klieneberger and Dienes is that the large bodies reproduce the small forms. In L1 the growth of the small forms from the large bodies has been observed (13) in the same culture which was used later in the filtration experiment. Morphological observations give support to the supposition that a similar process occurs in other cases. The large bodies in some L cultures are

filled with granules similar in size, staining and arrangement to the growing small forms (Photograph 18, Plate I). These granules are often present in masses after the disintegration of the large bodies. All transitional forms present from the smallest to the largest in cultures and organisms of varying size may be seen in short chains. It has thus far been impossible to observe directly the growth of the granules produced in the large bodies because very few of them will develop into a colony on the surface of the agar. The fact that the large bodies derived from bacilli reproduce bacilli and L forms gives support to the supposition that the large bodies are reproductive also in the L cultures.

Both the small and the large forms in the L cultures are soft and fragile. One of the characteristic differences between the bacterial and L colonies is their reaction to a slight mechanical injury. When a coverslip is placed on the agar, the individual bacilli are often dispersed on the agar in the area surrounding the colonies but their shape remains intact. The L colonies behave like a mucoid mass. Their edges are drawn into thin sheets or filaments in which the individual organisms are not recognizable. The staining of the L forms is slight in dry preparations. The young growth is deeply stained in wet stained agar preparations with the same hue as bacteria.

Except for their small size, all characteristics of the L forms including their fragility occur in pleomorphic bacteria. Not any single property but a characteristic combination of properties differentiates the L forms from the usual bacteria.

The 3B colonies observed in Proteus, Salmonella and Hemophilus differ from the other L colonies by their faster and more abundant growth. Like the 3A forms, they start to grow as small granules penetrating into the agar. The granules stain more deeply, have a sharper contour and have a greater tendency to swell into large bodies (Photograph 11, Plate I). Sometimes, even in very young colonies consisting of a few dozen organisms, the majority of these are large bodies and only a few small granules are seen at the periphery. The swelling occurs not only on the surface of the medium but also within the agar and thus gives a distinctive appearance to the youngest 3B colonies. In older colonies after the initial invasion of the agar, growth seems not to depend on direct multiplication of the small forms but on the cycle of swelling to large forms and the disintegration of these again into small forms.

The most important difference between 3A and 3B colonies is in their relative stability. If penicillin is eliminated, the 3B colonies reproduce the bacilli *en masse* within a few hours. A return of the bacilli in the 3A colonies was observed in only three species (*Proteus* sp., *Bacteroides* sp. and *S. moniliformis*), and the bacilli reappeared only after prolonged incubation and never *en masse*. Whether the 3A cultures in other species completely lost the ability to return to bacillary forms remains undecided. The gradual loss of this ability was observed in L1 and in the L forms of Proteus. Growth in liquid media, which seems to be a prerequisite for the return of bacteria, was observed only in *Salmonella* sp., in addition to the three groups of bacteria already mentioned. This genus is the only one in which 3A L type cultures developed in liquid media and were stable. The observations with the other L forms in which the return of bacteria was not observed carry little weight because they were not thoroughly studied.

The large bodies of the 3B colonies and those developing directly from bacteria present entirely similar reproductive processes. Both reproduce either L forms or bacteria. Neither of them grows in broth containing penicillin. On the surface of agar, if penicillin is present, they develop into 3A colonies and only rarely into 3B colonies, while in pour plates they more often produce 3B colonies. In the 3B colonies, the large bodies produced from the bacteria multiply without a change in their potential development. 3B colonies have been observed thus far only in the presence of penicillin and on agar medium. They probably are not observed more often because they are less stable than either the bacilli or the 3A colonies, and it is difficult to realize the conditions necessary for multiplication in such forms.

The properties of 3A and 3B L type colonies suggest that under certain conditions, bacteria are able to multiply limitlessly, but their morphology changes and their metabolic rate decreases. This change apparently occurs in two steps: (a) Development into large bodies and 3B L type colonies. From these the bacteria reappear readily. (b) Growth of 3A L type colonies which develop more slowly, whose growth requirements are more specific and from which the return to the usual bacterial form is not often seen. The source of the difficulty of recognizing the L forms in the natural habitat of bacteria and of assessing their significance may be that the 3B forms are the true variant and in our cultures they resume the bacterial form.

Our knowledge of the structure of bacteria was considerably advanced by the study of their chromatinic structures. The chromatin is present in granules which probably exert nuclear functions and indicate the cells or living units from which the bacteria are built (1, 70, 73). The bacterial filaments, like those of molds, contain many cells arranged in a row and enclosed in a common cell wall. The chromatin granules are stained clearly in the bacterial filaments of *Bacteroides* strain 132 (32) or of *Proteus* sp. (85). When the large body begins to develop, the chromatin granules instead of extending linearly slide by each other and are arranged tridimensionally. The well-developed large body contains chromatin granules similar to those of a bacterial filament and seems to be analogous to the filaments, with the difference that the spatial arrangement of the cells changes (Photographs 4 and 5, Plate I). The chromatin granules are distributed into the bacteria which develop from them (Photograph 6, Plate II). When large bodies disintegrate into bacilli, segmentation, separating pre-existing units as in the usual bacterial filaments, can occasionally be seen (Photograph 2, Plate II). The swelling into large bodies is a growth process, and it is certainly not the consequence of osmotic changes.

Another structural property of the large bodies is apparent in Proteus. Under certain conditions such as, for example, when the spreading filaments of Proteus are transferred to tap water, the large bodies are not produced by gradual swelling, but the content of the filament flows out from the membrane within a few minutes and produces a large body (18). The filament meanwhile visibly collapses. This process was described by Dienes (18), and was also observed by others (37, 89). The large body so produced has no membrane comparable to bacterial membranes, although it is elastic and resists considerable mechanical

injury (18). When the large body develops into bacilli, rigid bacillary cell walls develop only after the cells return to their usual bacillary form. When the large bodies develop into L forms, small soft granules grow out from the large body, and continue to grow in this form. It is apparent that Proteus can survive devoid of the usual bacterial membrane, and the L form seems to be the growth of the elementary units of bacteria without a membrane.

Boivin *et al.* (1) developed an efficient method for the staining of the chromatin in bacteria by extraction of the ribonucleic acid from the protoplasm with the help of ribonuclease. Tulasne applied this method (85, 86) to the study of L transformation in Proteus. The large bodies presented the structure already mentioned and the small L forms consisted mainly of desoxyribonucleic acid granules with a narrow fringe of protoplasm. Boivin, Tulasne *et al.* observed also that under the influence of penicillin which is so effective in producing L forms, the desoxyribonucleic acid content of bacteria markedly increases, and in contrast, the ribonucleic acid content decreases (1). Glycine which produces L transformation is also connected with nucleic acid metabolism. Tulasne (89) is inclined toward the consideration that in the L forms the metabolism is concentrated to the synthesis and preservation of the most important part of the cell, the nucleus, without regard to most of the other components of the cell. The change of structure and slowing down of the metabolism may secure the survival of the bacteria in conditions in which the usual bacterial form cannot survive.

It is too early to say whether the considerations just described will give the final answer concerning the nature of L forms, but they are of importance because they give a reasonable explanation for the transitions between the L forms and bacteria.

It is not known whether the chromatin granules have to pass through a change before they can grow out as L forms. Dienes and Smith (31) observed that some of the chromatin granules in the large body grow to a larger size, and the L forms apparently arise from them. However, it is possible that the larger chromatin-staining structure represents an L form already growing within the large body. Klieneberger (52) recently described the origin of L forms from the fusion of two of the elementary living units which are indicated by the chromatin granule within the bacilli. These granules, as in a sexual process, participate in the fusion. These observations if substantiated would be of the greatest interest. However, in the opinion of the reviewers, the descriptions and illustrations of this process are not conclusive. Furthermore, the observations deal with bacteria made pleomorphic by various injurious agents and not with true L type cultures.

Smith, Hillier and Mudd (81, 82) studied pleuropneumonia-like organisms of human origin and the bacillary and L forms of *Bacteroides* strain 132 with the electron microscope. The main difference observed between the bacilli and the two other groups of organisms was in the character of the cell membrane, which was more delicate in the latter than in the bacilli.

Although the rigid bacterial membrane is lacking in the pleuropneumonia-like organisms and L forms, a definite cell boundary is clearly seen in stained

preparations and with electron microscope, both in the small organisms and the large bodies. The boundary between the individual organisms inside the large bodies, regardless of whether they develop into bacilli or into L forms, only becomes visible when the smaller forms have segmented from each other. The L forms once separated do not coalesce with each other to form larger ones. The large body is derived from a single small organism which probably undergoes multiplication remaining inside of a common envelope.

The apparent connection between rough growth and L transformation should be mentioned. Concentrations of penicillin too small to inhibit bacterial growth will induce the development of long filaments and the colonies then acquire a rough appearance. Somewhat larger amounts will induce swellings in the bacterial filaments. Sublethal concentrations of various toxic substances, $HgCl_2$, for example, produce similar changes. A slight interference with the usual reproductive process produces growth as filaments, while a greater disturbance may change the spatial arrangement of the undivided growth. The large bodies and L forms may represent the limit of the transformation, the first step of which is rough growth.

SEROLOGICAL AND METABOLIC PROPERTIES OF L TYPE COLONIES

In contrast to the developmental and morphological characteristics, the serological and metabolic properties of L type cultures isolated from different species differ considerably from each other and resemble those of the parent organism. The serological properties of the L forms of Streptobacillus (6, 41, 48, 49), Proteus and Salmonella were studied (34, 93). In most strains, the serological similarity between the L forms and bacillus appeared to be complete, but in some, differences were present in the titers of the sera or were demonstrated by agglutinin absorption (table 2).

Agglutination occurs with the L form usually in low titer and the turbidity of the emulsion increases considerably with high concentrations of the antiserum. In that respect, the reaction is similar to the precipitin reaction. The filtered extract of the agar cultures of *Proteus* L forms gave excellent precipitin reactions. Similar reactions were not obtained in *Salmonella* sp.

It would be of great interest to study the antigenic structure of the L forms compared with those of the bacteria. The same can be said concerning the study of the metabolic activities and enzyme systems of L forms. Such studies may prove more important for the understanding of the nature of the L transformation than the study of the morphology of L forms. It may also help in understanding the effect of penicillin on bacteria, since there is an abrupt change in the reaction of bacteria to penicillin when they grow in the L forms. Changes produced in the metabolism of staphylococci and of Proteus by penicillin were studied recently by Ebel, Vendrely and Tulasne (35, 36).

CONDITIONS FAVORING THE TRANSFORMATION INTO L FORMS

The spontaneous development of L forms is always preceded by autolytic processes in the bacterial cultures (13, 32). Multiplication in the usual bacterial

forms is arrested and either all or a certain percentage of the bacteria swell to large forms. This process seemingly is caused in many cases by the accumulation of metabolic products. This was apparent in broth cultures of *Bacteroides* strain 132 and in agar cultures of the Clostridium described above. Spontaneous pleomorphism in a usually non-pleomorphic species is probably the result of a temporary disturbance in the metabolic processes of the bacteria. This conclusion agrees with the observation that pleomorphism and swelling of bacteria into large bodies occur under various conditions interfering with usual growth. This subject has a large literature which has been reviewed repeatedly (44, 79, 92).

TABLE 2

Summary of bacillary and L agglutination reactions

ANTISERA	ANTIGENS							
	S. typhosa		*S. typhimurium*		*Proteus #52*		*Proteus #3*	
	Bacillus	L	Bacillus	L	Bacillus	L	Bacillus	L
S. typhosa								
bacillus	1:2500	1:32	1:80*	0	0	—	0	0
L	1:2500	1:32	1:80*	—	0	0	0	0
S. typhimurium								
bacillus	1:320*	0	1:2500	1:80	0	—	1:40*	0
L	1:160*	1:16*	1:640	1:80	0	0	1:10*	0
Proteus #52								
bacillus	—	—	0	0	1:5120	1:40	1:1280	0
L	—	—	0	0	1:40*	1:640	1:40*	0
Proteus #3								
bacillus	—	—	0	0	1:20*	0	1:5120	1:640
L	—	—	0	0	0	0	1:5120	1:160

* indicates slight reaction.
0 indicates negative reaction.
— indicates not done.

Toxic substances in an otherwise appropriate medium often induce the development of large bodies. Lowering or raising of the pH of the medium (72) or absence of a required nutrient may have a similar effect. For example, streptomycin-dependent bacilli may swell into large bodies when transferred to a medium free of the antibiotic (69). Absence of growth factor "x" produces the effect in certain of the hemophilic bacteria. High concentrations of organic compounds such as glycine (33) *dl*-methionine, *l*-phenylalanine (28), caffein or raffinose (56), light metal salts, and sublethal doses of certain heavy metal salts induce swelling in almost all gram negative bacilli and occasionally in gram positive cocci and bacilli. The effect of different bacteriostatic and bactericidal agents varies considerably in this respect; and simple exhaustion of the medium in old cultures does not result in the swelling of bacteria. Various other factors such as exposure of the spreading Proteus filaments to cold (18), and typhoid bacilli to antibody and to phage, may induce transformation into large

bodies. The effect of penicillin is the most remarkable because it occurs in many species and over a wide range of concentrations.

The tendency to become swollen into large bodies is variable among bacteria; almost all will become swollen under certain conditions. It is interesting that certain species, for example, the alpha hemolytic and non-hemolytic streptococci, occasionally develop large bodies under apparently normal cultural conditions, but they are uninfluenced by agents usually effective in producing large bodies in other species.

The large bodies formed under such variable conditions often are viable, and either return to bacteria or grow into L forms. Whether all large bodies are of similar nature and should be regarded as the first step in the development into L forms cannot be decided. The development of L forms was observed under variable conditions such as spontaneous autolysis, exposure to penicillin, glycine, carboxylmethoxylamine, refrigeration, tap water, antibody and to bacteriophage (table 1). Varying numbers of the large bodies under these conditions also returned to the bacterial form. This indicates that the nature of the large bodies is probably the same whether or not L forms are produced. The various agents inducing L transformation seem to have in common only that they interfere with the normal reproduction and permit the growth of L forms. This is apparent in the case of bacteriostatic chemicals, the ability of which to induce the growth of L type colonies seems to depend upon their relative toxicity for the bacteria and the L forms (33). In contrast with its effect on the bacteria, penicillin has no toxicity for the L forms; carboxylmethoxylamine and glycine are slightly less toxic for the L forms than for the bacteria and aureomycin and chloramphenicol are about equally toxic for both. In the presence of the latter two antibiotics, the L forms start to grow but soon die out.

THE POSSIBLE FUNCTIONS OF L FORMS OF BACTERIA

Transformation into L forms is produced apparently always by an injury to the bacteria. It involves a change in the structure and metabolism of bacteria. Is this transformation a purely degenerative process or is it a *useful reaction* serving a definite purpose? Are the L forms specialized growth forms, or are they accidental products of outside influences which are kept artificially in growth in our cultures and would be eliminated under natural conditions? Heilman suggested that the L forms are probably degenerative (41, 42). Only the discovery of useful functions of L forms would help to answer these questions. Unfortunately, our information is sparse and inconclusive on these matters.

That L forms may have a genetic function accomplishing the rearrangement of hereditary properties of the strains has been considered. Certain characteristics of *Proteus sp.* are especially helpful in studying this question. The bacilli are easily regained from the L forms and changes in the individuality of the strains can be easily determined (19). No change was observed in the strain specificity of *Proteus* strains recovered from large bodies or from L forms (19, 24). This was the case even when the cultures were kept in the L forms for several months, and when L forms of several *Proteus* strains were grown for

several days in mixed cultures (28). However, the development of large bodies in certain cases presents morphological similarities to the sexual processes of fungi. It is often apparent that the large bodies in *Bacteroides* strain 132 develop from the adjoining ends of two segments of the bacillary filaments (80) (Photograph 1, Plate II). In certain cases the filaments of *E. coli* and *S. typhosa* first produce an open loop and the large body develops from the fusion of the loop (93). Observations of this type have been described repeatedly (59, 60). It is unlikely that such complex processes are purely degenerative, but their significance is obscure at present. Dienes (17) called attention to morphological similarities between the L type cultures and the "haploform" yeast cultures of Winge (94).

It is remarkable that passage of the bacilli through L forms which are extremely resistant to penicillin does not increase the resistance of the bacilli to the antibiotic. The only change in the properties of bacilli regained from the L form is the marked pleomorphism which was observed in *Bacteroides* sp. and *Salmonella* sp.

The L forms have an increased resistance compared with the bacteria in autolyzed cultures and in the presence of penicillin and of some other bacteriostatic chemicals. Whether this means that they are resistant forms, resistant not to drying and heat as spores, but to certain chemical injuries, is not certain, because all our observations have been made under artificial conditions. We have no evidence that the L forms play any role in the natural environment of the bacteria. It is not known, for example, whether penicillin has any importance in the competition between molds and bacteria (38).

When the bacteria were transformed into L forms, their pathogenicity had disappeared in all cases. This was observed in *S. moniliformis* (13, 41), *Flavobacterium* (13), and *Salmonella* (93) strains highly pathogenic for mice. *S. moniliformis* recovered from L1 continued to be pathogenic, and fresh L1 cultures may revert in mice and produce a bacterial infection. The L type cultures are similar in many respects to the pleuropneumonia group, and if they are similar also in their pathogenic properties, it may be difficult to observe these properties. The pleuropneumonia-like organisms are strictly host-specific and their virulence decreases rapidly under cultivation. Cultivation of L forms from pathological processes may be unsuccessful because they are very sensitive to the inhibitory effects of fresh tissue. Bacteria cultivated from pathogenic processes occasionally show a temporary pleomorphism like the bacteria regained from L cultures. Whether this indicates that the bacteria recently regained their usual form and then were present in the host in the L form cannot be decided at present. An observation suggesting the pathogenicity of Bacteroides L forms in a human infection was described by Dienes and Smith (32).

Pleuropneumonia-like organisms cultivated from urinary infections often are indistinguishable morphologically from L type cultures (29). Such organisms often can be cultivated from the mucous membranes following clinical administration of penicillin. Whether these represent L forms of bacteria developing *in vivo* or a part of the normal flora is as yet undetermined.

Even if we have no positive information on the significance of the L forms, the survival of bacteria in such forms can but arouse our interest. If they have no other significance, they offer an opportunity for basic studies on the structure and metabolism of bacteria. They deserve interest from the viewpoint of general biology because they belong to the smallest living forms capable of independent life.

THE CONNECTION BETWEEN THE BACTERIA AND THE L FORMS AND THE SIMILARITY OF PLEUROPNEUMONIA-LIKE ORGANISMS AND L FORMS

The consideration of the nature and functions of the L forms discussed thus far presupposed that these forms are genetically identical with the bacteria. The reluctance to accept this identity arises mainly from general considerations. It means a radical change in our views on the morphology and variability of bacteria and similar claims, often made in the past, were never supported by convincing evidence. The great importance of the discovery of L1 was that it could be isolated and studied at will. A large part of the observations on the L form are of similar nature; they are reproducible and they can be made with simple and controllable techniques. No investigator who has studied the L forms questions the validity of the basic observations. The problem is only in the interpretation of the observations: Does the information which we possess give sufficient evidence for the identity of L forms and bacteria, or does the alternate explanation that they are parasites or symbionts of the bacteria retain some probability?

As in the description of the properties of the L forms, we have to base our conclusions on the most thoroughly studied groups of bacteria, the first five entries in table 1. The pertinent observations are the following: a. The constant presence of the ability in certain species to produce L forms. b. The observation of the growth of L forms from bacteria. c. The serological and metabolic similarities between L forms and their corresponding bacteria, and the absence of such similarities between the various L forms. d. The reappearance of the bacilli in the L cultures. e. The properties of 3B colonies.

Every strain of *S. moniliformis*, *Proteus* and *Salmonella* properly examined produced L type colonies. The strains of *Bacteroides* and *Flavobacterium*, which in cultivation lost the ability to produce L forms spontaneously, continued to do so in the presence of penicillin. The attempts of Klieneberger to eliminate the L forms from *S. moniliformis* were not successful (46). A similar experiment was made by the reviewers on *Salmonella* sp. (28). Five strains were isolated from individual colonies of a culture of *S. typhimurium*, and each strain was transplanted six times also from individual colonies. It is probable that the cultures at the end were descendants of single bacilli. Twenty-four colonies were transplanted from these strains on penicillin plates and L forms grew equally well from all of them. The observation that all viable bacilli swell to large bodies in the presence of penicillin in the three species mentioned shows that the tendency to L transformation is present in all bacteria.

The return of the bacilli from the 3A colonies was observed in three of the

four intensively studied groups of bacteria. In Salmonella, where the 3A colonies have proved to be stable thus far, the bacilli were recovered from the 3B colonies. Albert Sabin, in several discussions at meetings of the Society of American Bacteriologists, expressed the opinion that decisive evidence for the growth of bacteria from the L forms would be obtained by filtration of the L cultures through gradocol membranes and by regaining the bacteria from the fraction of the smallest size. At present it would be technically difficult to make this experiment because our cultural methods are not good enough to grow the organisms from the filtrates. It is desirable to study the filtrability of the L forms. However, the evidence for the identity of L forms and bacteria can be based only on observations which are available at present.

The most striking evidence for the identity of L forms and bacilli is furnished by the behavior of 3B colonies. If cultures with similar morphology which are stable in the absence of penicillin were not known, the 3B colonies would be regarded simply as a modification of the bacterial morphology by penicillin and their identity with the bacilli would not be questioned. If we admit the identity of 3B colonies with the bacilli, the case is really decided for all L forms. Once it is admitted that the bacilli can grow with the characteristic morphology of the L forms in one case, there is no reason to object to this conclusion on the basis of general considerations. It should also be considered that the morphological differences between the L forms and bacteria are not so great as they originally appeared. According to findings already discussed, there is transition between the structure of bacterial filaments, the large bodies and the L granules. When the whole process of transformation is considered, the structure of the L forms seems to derive by relatively simple steps from the structure of the bacteria.

Our information on the other bacteria represented in tables 1B and 1C is incomplete. It already has been pointed out that all information which we have on these L forms indicates that they are essentially similar to the species most thoroughly studied. No observations were made with them suggesting that they are symbionts or parasites.

The only positive support for the symbiotic or parasitic nature of the L forms is their morphological similarity to a group of microorganisms, the pleuropneumonia group, which is thought to be different from bacteria. Klieneberger tried to get more evidence for this supposition. The crucial proof for the parasitic nature of L1, transfer of the supposed parasite from one culture to another, could not be made with *S. moniliformis* nor with the later-studied bacteria, because no culture could be obtained free from the L forms. Klieneberger (48) tried but was unable to produce an artificially symbiotic relationship between the L1 and pleuropneumonia-like organisms and bacteria. During the sixteen years since the L1 has been known, no observations have been recorded which would give positive support to the parasitic theory and which would present a difficulty for the identity of the L forms and bacteria. In the meantime, many observations of various types have accumulated which uniformly support their identity. The parasitic theory was first considered and it has been abandoned because it did not agree with the findings. This is apparent if the be-

havior of bacteriophage and the L forms is compared. A strain of bacterium can be freed from the phage and again infected with it. The phage survives and multiplies only in the bacteria; its metabolism and serological properties are widely different from those of the bacteria. It is generally accepted that the bacteria cannot be regained from them. The observations made with the L forms are different in all these points.

It is not very important that the identity of the L forms and of the bacteria be regarded as proven now, but it is very important that it be regarded as probable. The L forms have at present no practical importance, but they may be the starting point of much new information on bacteria. The direction of our thoughts depends on what seems probable to us, and the future study of the L forms will be greatly influenced by the views which we form of their nature.

The similarity of the L forms to the pleuropneumonia-like organisms is one of their most interesting properties. It is the cause of the controversy concerning the origin of the L forms, but if we admit that these forms are derived from bacteria, their similarity to an independent group of organisms indicates that transformation into L forms is under certain conditions an adaptive process.

A certain amount of similarity between the L forms and the pleuropneumonia group is generally admitted. The question is whether these two groups of organisms can be differentiated from each other, and if not, whether they should be regarded as essentially similar in nature. A concrete example will illustrate the problem. Organisms of the pleuropneumonia group usually can be isolated from the human throat and from the female genitals (29). These organisms were studied by several authors and their classification with the pleuropneumonia group is not questioned. No connection of these organisms to bacteria is apparent. The reviewers believe that if the 3A L forms of Salmonella which do not return into bacillary forms were cultivated from similar sources, they would be regarded also as members of the pleuropneumonia group. There is nothing in the appearance of the colonies or in the morphology of the organisms to differentiate them from this group. Only the study of the serological and metabolic properties would identify them as *Salmonella* L cultures.

The photographs in Plate II illustrate this similarity. They were made from organisms isolated from human genitals. The appearance and structure of the colonies, the small size of the organisms and their arrangement in the growing colonies, and their swelling to large forms are very similar in both groups of organisms. The small granules are produced in the large forms and grow out of them in a similar way (Photographs 26, 27, 28, Plate II). The physical properties of the organisms such as softness, fragility and staining reactions are similar in both groups. Furthermore, the *Salmonella* 3A cultures, like those of the pleuropneumonia group, require for growth a complex medium with fresh animal protein; they are equally insensitive to the action of penicillin and equally sensitive to other antibiotics. Slow growth of the cultures is characteristic both of the L forms and of the pleuropneumonia group.

It is a matter of observation that the characteristics just enumerated define a well-circumscribed group of organisms. It is rarely questioned whether or

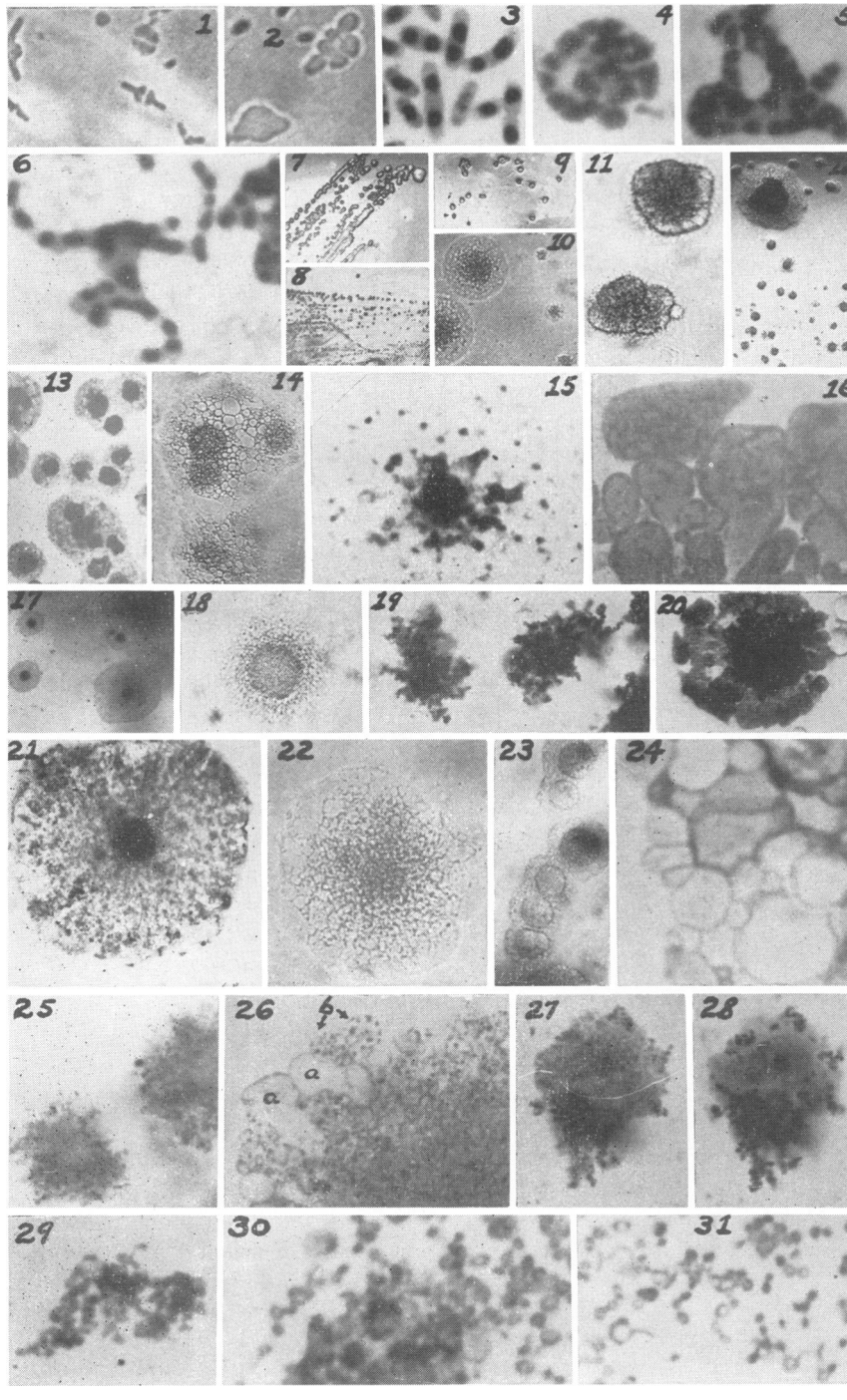
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PLATE II

not a given organism belongs to it. The basic difference between these organisms and bacteria may be in morphology, in metabolism or in some special functions which the L forms perform. Until the nature of this basic difference is known, the definition of the group is purely empirical and the lesser or greater importance of their various properties remains questionable.

PLATE II

Plate II illustrates certain structural properties of the large bodies, the appearance of various L colonies with low magnification and pleuropneumonia-like organisms.

Photographs 1–6 were made from strain 132 of Bacteroides.

1. Development of the large bodies from two segments of the bacterial filaments. Broth culture after 3 hours' incubation without penicillin. Unstained. × 800.

2. Segmentation of a short filament with swelling into bacilli after transfer from broth to agar. A smaller body with similar shape remained unsegmented. Unstained. × 2,000.

Photographs 3–6 showing the chromatin granules were made from broth cultures. They were transferred to agar and agar fixation preparations were made at once. The preparations were stained with Giemsa solution and slightly decolorized with eosin. × 3,000.

3. Young broth culture. The bacilli show one or two chromatin bodies.

4. A large body with arrangement of the chromatin bodies at the periphery, from 24-hour-old broth culture.

5 and 6. Growth of the large bodies into bacilli two hours after transfer into fresh broth. The structure corresponds to those in No. 27 of Plate I. The chromatin granules are distributed in the bacterial filaments.

Photographs 7–20 show the L cultures in different species as they appear with the hand lens and with low magnification.

7. One-day-old culture of *S. moniliformis* photographed with reflected light. Natural size.

8. Three-day-old culture of L1 photographed with reflected light. × 2.

9. A similar photograph of L colonies of Bacteroides three days old. The sharp contour and ring-like appearance are characteristic of L colonies of larger size. × 2.

10 and 11. Different appearances of fully developed L1 colonies. Unstained. × 100.

12. 3A and 3B L colonies of Salmonella on penicillin agar after 2 days' incubation. Unstained. × 30.

13. 3A L colonies of Salmonella from a stained agar preparation. In transplants colonies develop to larger size than in the plates inoculated with bacilli. × 100.

14. L colonies of *S. typhosa* after three days' growth. The foamy appearance is the result of the vacuolization of large bodies. Unstained. × 100.

15. L colonies of Bacillus 2 spreading on serum agar with many daughter colonies after 7 days' incubation. Dry stained agar. × 200.

16. Large bodies on the surface of the L colony shown in Number 20. Wet stained agar. × 900.

17 and 18. L colonies of Bacillus 2 on hard agar without serum after three days' incubation. The colonies remain small on nutrient agar (No. 17); they are larger on starch agar (No. 18). Both unstained. × 100.

19. Young L colonies of Clostridium branching out into the agar after 2 days' incubation. A similar growth is characteristic of L colonies. Wet stained agar. × 200.

20. Same culture as Number 19. A colony with a well-developed periphery of large bodies. Wet stained agar. × 200.

Photographs 21–31 illustrate the structure and morphology of pleuropneumonia-like colonies isolated from human genitals.

21, 22 and 23. Various appearances of fully developed colonies with low magnification.

It is the impression of the reviewers that it is impossible to give a reasonable definition of the pleuropneumonia group which would exclude the L forms. There is no place in this review to discuss the long and complex development of our information on the morphology of the pleuropneumonia group. Sabin's definition of the pleuropneumonia group (75), which is most widely known in the United States, is based essentially on the properties which were enumerated above, only a different technique is recommended for the examination of the cultures. He included the L1 in the pleuropneumonia group with the reservation that the size of the smallest reproductive unit should be determined by filtration and should be less than 0.2 micron. Small size is a characteristic of the pleuropneumonia group, but it seems arbitrary to admit or exclude organisms from a morphologically well-defined group according to an exact size. If the L1 is included in the pleuropneumonia group, the L forms of other bacteria also belong in it.

Klieneberger's views on the similarity of L1 and the pleuropneumonia group have been accepted by most authors without comment. The only dissenting opinion was expressed by Oerskov (67) and his pupil, Freundt (37). According to them, the morphology of the L forms is essentially bacterial and there is not even a remote similarity between them and the pleuropneumonia group. Their idea of the morphology of the pleuropneumonia group is different from that of most authors. The following passage is quoted by Freundt from a paper of Oerskov (68): "The elementary, initial or terminal bodies are small round or oval, which to begin with grow out into an undivided, ramified branching my-

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Number 21, wet stained agar preparation, × 100; Number 22, unstained, × 150; Number 23, unstained, × 100.

24. Large bodies at the edge of a colony. Wet stained agar. × 2,000.

25. Two young (2 days old) colonies. This photograph corresponds to Number 4 of Plate I. The focus is set beneath the surface of the agar. The surface of the colonies consists of large bodies. Wet stained agar. × 900.

26. Large bodies on the edge of a colony partly empty (a) and partly filled with small forms (b). Same strain as the colony shown in No. 21. Wet stained agar. × 900.

27 and 28. A single large body transferred from the same culture as No. 26 after incubation overnight. In No. 27, the focus is set on the surface. The large body is considerably increased in size and is filled with small granules, strands of which grow out of it. The growth of the granules from different parts of the large body is more clearly visible in No. 28 where the focus is set lower. Wet stained agar preparations. × 900.

29. A young spreading colony of the same strain as in No. 28 after 24 hours' incubation in a dry stained agar preparation. The individual organisms are not clearly visible but the similarity of the structure to the L1 colony in Nos. 20 and 21 of Plate I is apparent. × 2,000.

30. Impression preparation of young culture. The organisms are similar to those on the L colonies. They are very small, often in pairs and gradually enlarging. Agar fixation preparation. × 3,000.

31. Smear from a broth culture of a strain of a pleuropneumonia-like organism. The few curved filaments are artefacts produced by deformation of the small forms. Giemsa staining. × 2,400.

Photographs 18 and 26 of Plate I and photograph 2 of Plate II were formerly published in the Journal of Bacteriology and are reproduced with the permission of the publishers (Vol. 59, p. 768; Vol. 54, p. 235; Vol. 48, p. 147).

celium and this divides without demonstrable septa in the elementary bodies." Freundt continues: "The large swollen forms, which in fairly old cultures occupy the pale peripheral zone, are regarded by Oerskov (and justly so, as far as I can judge) as involution forms." These statements disagree with the observations of Klieneberger and of the reviewers. Sabin (75) also fails to mention the development of branching filaments in murine strains. Oerskov (65) studied originally the bovine strains which produce filaments. He relies mainly on the microscopical study of unstained agar cultures which does not give sufficient resolution. The presence of branching filaments in the agar cultures certainly is not a characteristic of the pleuropneumonia group of organisms, and their absence does not differentiate the L forms from the pleuropneumonia group.

Our knowledge of the structure of microorganisms compared with the higher plants and animals is very incomplete, and our suppositions on the derivation and relationships of different bacterial forms are always more or less uncertain. With these reservations in mind, it is difficult to believe that the similarities between the L type cultures and the pleuropneumonia organisms extending to so many points are accidental. These characteristics represent a simplification of the bacterial structure, and when bacteria are transformed into L forms, they assume these properties. It is very likely that the origin of a similar complex group of properties is the same in the pleuropneumonia group. This group probably descended at some time from the bacteria and became stabilized to live in this form. If this supposition is correct, though the pleuropneumonia group is well-defined, it is not analogous to an order or family in the natural system but represents a growth form which various bacteria can take up. The pleuropneumonia group is in the same position as the *Fungi imperfecti*; they form a morphological group of miscellaneous origin, the distribution of which in the natural system is at present impossible.

The speculations just discussed suggest further speculations concerning the the development of the viruses. Two aspects of the problem should be distinguished. One is the phylogenetic development of the viruses at some time in the past. It is a widely held supposition that some of the viruses developed from other microorganisms by gradual loss of properties as a consequence of parasitism. The discovery of the L forms and their properties gives support to this idea. The other aspect of the question is whether viruses develop from bacteria at the present time. The observations made thus far with the L forms offer no evidence for this supposition. We have no information on the role of L forms in the pathogenic action of bacteria. Well-controlled, positive observations on this point would be of great interest. Until they are made, speculations have little value.

It is outside the scope of this review to discuss the possible relationship of the L forms to the so-called filtrable forms of bacteria, to bacterial gonidia and to various pleomorphic forms described in the literature. Klieneberger suggested in a recent paper (54a) that some of these are probably analogous to the L forms. She feels especially justified to identify the filtrable G forms of Hadley (40) with the L forms, and she regards the discovery of the latter as confirmation of the older observations. The reviewers do not agree with Klieneberger's con-

clusions. The differences between the properties of L forms and the description given of filtrable forms are marked. In Hadley's experiments, the G forms grown from unfiltered cultures were small colony variants of bacteria. The properties of G forms grown from filtered cultures also had no similarities to those of L forms. The appearance of the cultures, their microscopic morphology and their physical properties were different. The G forms grew without animal serum, preferably in broth, and the cultures survived for years. They were easily filtrable. Such observations have never been made with the L forms of Salmonella and Shigella. Bacteria reappeared in the G cultures very rarely and only by a long gradual development, and doubt remains as to their identification with the parent strains. The reviewers have never observed cultures corresponding to Hadley's descriptions, and in their opinion his observations as they were reported indicate no relationship between the L forms and the so-called filtrable forms of bacteria. Unless a technic is devised by which the filtrable forms can be produced regularly so that their properties may be studied, their very existence remains questionable.

REFERENCES

1. BOIVIN, A., TULASNE, R., VENDRELY, R., AND MINCK, R. 1947 Le noyau des bactéries. Cytologie et cytochemie des bactéries normales et des bactéries traitées par la penicilline. Arch. Sci. physiol., **1**, 307–323.
2. BORREL, DUJARDIN-BEAUMETZ, JEANTET AND JOUAN. 1910 Le microbe de la péripneumonie. Ann. Inst. Pasteur, **24**, 168–179.
3. BROWN, T. McP., AND HAYES, G. S. 1942 Isolation of microorganisms of the pleuropneumonia group from apparently pure cultures of the gonococcus (*Neisseria gonorrhoeae*). J. Bact., **43**, 82–83 (Abstr.).
4. BROWN, T. McP., AND NUNEMAKER, T. C. 1942 Rat bite fever. Bull. Johns Hopkins Hosp., **70**, 201–327.
5. DAWSON, M. H. Personal communication.
6. DAWSON, M. H., AND HOBBY, G. L. 1939 Pleuropneumonia-like organisms as a variant phase of *Streptobacillus moniliformis*. Third Intern. Congr. Microbiol., Abstr. Communic., p. 21.
7. DAWSON, M. H., AND HOBBY, G. L. 1939 Rat bite fever. Trans. Assoc. Am. Physicians, **54**, 329–332.
8. DIENES, L. 1939 "L" organisms of Klieneberger and *Streptobacillus moniliformis*. J. Infect. Dis., **65**, 24–42.
9. DIENES, L. 1939 L type variant forms in cultures of various bacteria. Proc. Soc. Exptl. Biol. Med., **42**, 636–640.
10. DIENES, L. 1939 A peculiar reproductive process in colon bacillus colonies. Proc. Soc. Exptl. Biol. Med., **42**, 773–778.
11. DIENES, L. 1940 L type of growth in gonococcus cultures. Proc. Soc. Exptl. Biol. Med., **44**, 470–471.
12. DIENES, L. 1941 Isolation of L type of growth from a strain of *Bacteroides funduliformis*. Proc. Soc. Exptl. Biol. Med., **47**, 385–387.
13. DIENES, L. 1942 The significance of the large bodies and the development of L type of colonies in bacterial cultures. J. Bact., **44**, 37–72.
14. DIENES, L. 1943 Reproduction of bacteria from the large bodies of *Streptobacillus moniliformis*. Proc. Soc. Exptl. Biol. Med., **53**, 84–86.
15. DIENES, L. 1944 L type of growth in cultures of a hemolytic parainfluenza bacillus. Proc. Soc. Exptl. Biol. Med., **55**, 142–144.
16. DIENES, L. 1945 Morphology and nature of the pleuropneumonia group of organisms. J. Bact., **50**, 441–458.

17. DIENES, L. 1946 Complex reproductive processes in bacteria. Cold Spring Harbor Symp. on Quant. Biol., **11**, 51–59.
18. DIENES, L. 1946 Reproductive processes in *Proteus* cultures. Proc. Soc. Exptl. Biol. Med., **63**, 265–270.
19. DIENES, L. 1947 Further observations on the reproduction of bacilli from the large bodies in *Proteus* cultures. Proc. Soc. Exptl. Biol. Med., **66**, 97–98.
20. DIENES, L. 1947 The morphology of the L1 of Klieneberger and its relationship to *Streptobacillus moniliformis*. J. Bact., **54**, 231–237.
21. DIENES, L. 1947 Isolation of pleuropneumonia-like organisms from *H. influenzae* with the aid of penicillin. Proc. Soc. Exptl. Biol. Med., **64**, 166–168.
22. DIENES, L. 1948 Isolation of L type colonies from typhoid bacilli with the aid of penicillin. Proc. Soc. Exptl. Biol. Med., **68**, 589–590.
23. DIENES, L. 1948 The isolation of L type cultures from Bacteroides with the aid of penicillin and their reversion into the usual bacilli. J. Bact., **56**, 445–456.
24. DIENES, L. 1948 The influence of penicillin on the growth of Proteus cultures. Proceedings, Society of American Bacteriologists, 3–4.
25. DIENES, L. 1949 The development of Proteus cultures in the presence of penicillin. J. Bact., **57**, 529–546.
26. DIENES, L. 1949 Isolation of an L type culture from a Gram-positive spore-bearing bacillus. Proc. Soc. Exptl. Biol. Med., **71**, 30–33.
27. DIENES, L. 1950 Isolation of L type cultures from Clostridia. Proc. Soc. Exptl. Biol. Med., **75**, 412–415.
28. DIENES, L. Unpublished data.
29. DIENES, L., ROPES, M. W., SMITH, W. E., MADOFF, S., AND BAUER, W. 1948 The role of pleuropneumonia-like organisms in genito-urinary and joint diseases. New England J. Med., **238**, 509–515; 563–567.
30. DIENES, L. AND SMITH, W. E. 1942 Reproduction of bacteria from the large bodies of *B. funduliformis*. Proc. Soc. Exptl. Biol. Med., **51**, 297–298.
31. DIENES, L., AND SMITH, W. E. 1943 Chromatin structures suggesting a nuclear apparatus in the large bodies of *B. funduliformis*. Proc. Soc. Exptl. Biol. Med., **53**, 195–196.
32. DIENES, L., AND SMITH, W. E. 1944 The significance of pleomorphism in *Bacteroides* strains. J. Bact., **48**, 125–153.
33. DIENES, L., WEINBERGER, H. J., AND MADOFF, S. 1950 The transformation of typhoid bacilli into L forms under various conditions. J. Bact., **59**, 755–764.
34. DIENES, L., WEINBERGER, H. J., AND MADOFF, S. 1950 Serological reactions of L type cultures isolated from Proteus. Proc. Soc. Exptl. Biol. Med., **75**, 409–412.
35. EBEL, J., VENDRELY, R., AND TULASNE, R. 1950 Action de la pénicilline sur le métabolisme phosphoré et azoté du staphylocoque doré (*Staphylococcus aureus*). Compt. rend. Soc. Biol., **144**, 1413–1415.
36. EBEL, J., VENDRELY, R., AND TULASNE, R. 1950 Action de la pénicilline sur le métabolisme phosphoré et azoté du *Proteus vulgaris*. Compt. rend. Soc. Biol., **144**, 1415–1417.
37. FREUNDT, E. A. 1950 Observations on Dienes' L type growth of bacteria. Acta Path. Microbiol. Scandinav., **27**, 159–171.
38. GALE, E. F. 1949 The action of penicillin on the assimilation and utilization of amino-acids by Gram-positive bacteria. Symposia of the Soc. for Exptl. Biol., III, Selective toxicity and antibiotics. New York, Academic Press Inc., pp. 233–252.
39. GORDON, J., AND GORDON, M. 1943 Involution forms of the genus *Vibrio* produced by glycine. J. Path. Bact., **55**, 63–68.
40. HADLEY, P., DELVES, E., AND KLIMEK, J. 1931 The filterable forms of bacteria: I. A filterable stage in the life history of the Shiga dysentery bacillus. J. Infect. Dis., **48**, 1–159.
41. HEILMAN, F. R. 1941 A study of *Asterococcus muris* (*Streptobacillus moniliformis*). I. Morphological aspects and nomenclature. J. Infect. Dis., **69**, 32–44.

42. HEILMAN, F. R. 1941 A study of *Asterococcus muris* (*Streptobacillus moniliformis*). Cultivation and biochemical activities. J. Infect. Dis., **69**, 45–51.
43. KAUFFMANN, F., AND PERCH, B. 1947 On the occurrence of Proteus X strains in Denmark. Acta Path. Microbiol. Scandinav., **24**, 135–149.
44. KLIENEBERGER, E. 1930 Bacterienpleomorphismus und Bacterienentwicklungsgänge. Ergebn. Hyg. Bakt., Immunitätsforsch. Exper. Therap., **11**, 499–555.
45. KLIENEBERGER, E. 1934 The colonial development of the organisms of pleuropneumonia and agalactia on serum agar and variations of the morphology under different conditions of growth. J. Path. Bact., **39**, 409–420.
46. KLIENEBERGER, E. 1935 The natural occurrence of pleuropneumonia-like organisms in apparent symbiosis with *Streptobacillus moniliformis* and other bacteria. J. Path. Bact., **40**, 93–105.
47. KLIENEBERGER, E. 1936 Further studies on *Streptobacillus moniliformis* and its symbiont. J. Path. Bact., **42**, 587–598.
48. KLIENEBERGER, E. 1938 Pleuropneumonia-like organisms of diverse provenance: Some results of an enquiry into methods of differentiation. J. Hyg., **38**, 458–476.
49. KLIENEBERGER, E. 1940 The pleuropneumonia-like organisms: Further comparative studies and a descriptive account of recently discovered types. J. Hyg., **40**, 204–222.
50. KLIENEBERGER, E. 1942 Some new observations bearing on the nature of the pleuropneumonia-like organisms known as L1 associated with *Streptobacillus moniliformis*. J. Hyg., **42**, 485–497.
51. KLIENEBERGER-NOBEL, E. 1947 Isolation and maintenance of an L1-like culture from *Fusiformis necrophorus* (syn. *Bacterium funduliforme*, *Bacteroides funduliformis*). J. Hyg., **45**, 407–409.
52. KLIENEBERGER-NOBEL, E. 1949 Origin, development and significance of L-forms in bacterial cultures. J. Gen. Microbiol., **3**, 434–443.
53. KLIENEBERGER-NOBEL, E. 1949 On *Streptobacillus moniliformis* and the filterability of its L-form. J. Hyg., **47**, 393–395.
54. KLIENEBERGER-NOBEL, E. 1950 Methods for the study of the cytology of bacteria and pleuropneumonia-like organisms. Quart. J. Micro. Sci., 3rd Ser., **91**, 340–347.
54a. KLIENEBERGER-NOBEL, E. 1951 Filterable forms of bacteria. Bact. Rev., **15**, 77–103.
55. KLIENEBERGER, E., AND SMILES, J. 1942 Some observations on the developmental cycle of the organisms of bovine pleuropneumonia and related organisms. J. Hyg., **42**, 110–123.
56. KRITSCHEWSKI, I. L., AND PONOMEREWA, I. W. 1934 On the pleomorphism of bacteria. I. On the pleomorphism of *B. paratyphi* B. J. Bact., **28**, 111–126.
57. KUHN, PH., AND STERNBERG, E. 1931 Ueber Bacterien und Pettenkofferien. Zbl. Bakt., I, Orig., **121**, 113–161.
58. LAIDLAW, P. P., AND ELFORD, W. J. 1936 A new group of filterable organisms. Proc. Roy. Soc., London, B, **120**, 292–303.
59. MELLON, R. R. 1925 Studies in microbic heredity. I. Observations on a primitive form of sexuality (zygospore formation) in the colon-typhoid group. J. Bact., **10**, 481–501.
60. MELLON, R. R. 1925 Studies in microbic heredity. II. The sexual cycle of *B. coli* in relation to the origin of variants with special reference to Neisser and Massini's *B. coli-mutable*. J. Bact., **10**, 579–588.
61. MILLER, C. P., AND BOHNHOFF, M. 1947 Two streptomycin-resistant variants of meningococcoccus. J. Bact., **54**, 467–481.
62. MINCK, R. 1950 Obtention de formes L à partir des vibrion cholériques. Propriétés pathogène. Application à la protection des souris contre la maladie expérimental. Compt. rend. Acad. Sci., **251**, 386–388.
63. MORTON, H. E., SMITH, P. F., AND LEBERMAN, P. R. 1949 Symbiotic growth of pleuropneumonia-like organisms with bacterial colonies. Proc. Soc. Exptl. Biol. Med., **72**, 328–330.

64. OERSKOV, J. 1922 Method for the isolation of bacteria in pure culture from single cells and procedure for the direct tracing of bacterial growth on a solid medium. J. Bact., **7**, 537–549.
65. OERSKOV, J. 1927 Etude sur la morphologie du virus péripneumonique. Ann. Inst. Pasteur, **41**, 473–482.
66. OERSKOV, J. 1938 Zur morphologie der von G. Seiffert kultivierten Mikroorganismen. Zbl. Bakter., I, Orig., **141**, 229–232.
67. OERSKOV, J. 1942 *Streptobacillus moniliformis* and the morphology of its variants. Acta Path. Microbiol. Scandinav., **19**, 575–585.
68. OERSKOV, J. 1942 On the morphology of peripneumonia virus, agalactia virus and Seiffert's microbes. Acta Path. Microbiol. Scandinav., **19**, 586–590.
69. PAINE, T. F., JR., AND FINLAND, M. 1948 Observations on bacteria sensitive to, resistant to, and dependent upon streptomycin. J. Bact., **56**, 207–218.
70. PIEKARSKI, G. 1949 Zum Problem des bakterien Zellkerns. Ergebn. Hyg. Bakt., Immunitätsforsch. Exper. Therap., **26**, 333–362.
71. PIERCE, C. H. 1942 *Streptobacillus moniliformis*, its associated L1 form and other pleuropneumonia-like organisms. J. Bact., **43**, p. 780 (Abstr.).
72. REED, G., AND ORR, Y. H. 1923 Influence of H ion concentration upon structure: *H. influenzae*. J. Bact., **8**, 103–110.
73. ROBINOW, C. F. 1942 A study of the nuclear apparatus of bacteria. Proc. Roy. Soc., London, B., **130**, 299–324.
74. ROBINOW, C. F. 1945 Nuclear apparatus and cell structure of rod-shaped bacteria. Addendum to R. J. Dubos', The Bacterial Cell, in Its Relation to Problems of Virulence, Immunity and Chemotherapy. Cambridge, Harvard University Press, pp. 353–377.
75. SABIN, A. B. 1941 The filterable microorganisms of the pleuropneumonia group. Bact. Rev., **5**, 1–66.
76. SABIN, A. B. 1948 *Streptobacillus moniliformis*. Bacterial and Mycotic Infections of Man, edited by R. J. Dubos. Philadelphia, J. B. Lippincott Co., pp. 563–567; ibid., The pleuropneumonia group, pp. 568–575.
77. SANDER, F. 1938 Die atypischen Bakterienformen unter besonderer Berüchsichtigung des Problems bakterieller Generationswechselvorgänge. Ergebn. Hyg. Bakt., Immunitätsforsch. Exper. Therap., **21**, 338–493.
78. SCHAUB, I. G., AND GILBEAU, J. A. 1949 The occurrence of pleuropneumonia-like organisms in material from the postpartum uterus; simplified methods for isolation and staining. Bull. Johns Hopkins Hosp., **84**, 1–10.
79. SIEFFERT, G. 1937 Ueber das Vorkommen filtrabler Mikroorganismen in der Natur und ihre Züchtbarkeit. Zentr. Bakt. Parasitenk., I Abt., Orig., **139**, 337–342.
80. SMITH, W. E. 1944 Observations indicating a sexual mode of reproduction in a common bacterium (*Bacteriodes funduliformis*). J. Bact., **47**, 417–418 (Abstr.).
81. SMITH, W. E., HILLIER, J., AND MUDD, S. 1948 Electron micrograph studies of two strains of pleuropneumonia-like (L) organisms of human derivation. J. Bact., **56**, 589–602.
82. SMITH, W. E., MUDD, S., AND HILLIER, J. 1948 L-type variation and bacterial reproduction by large bodies as seen in electron micrograph studies of *Bacteroides funduliformis*. J. Bact., **56**, 603–618.
83. SMITH, W. E., AND ROPES, M. W. 1945 Bacteroides infections. An analysis based on a review of the literature and a study of twenty cases. New England J. Med., **232**, 31–37.
84. TOPLEY AND WILSON. 1946 Principles of Bacteriology and Immunology. 3rd ed. Baltimore, Williams & Wilkins Company, Vol. I, pp. 385–387.
85. TULASNE, R. 1949 Existence of L-forms in common bacteria and their possible importance. Nature, **164**, 876–877.
86. TULASNE, R. 1949 Cytologie des Proteus et ses modifications sous l'effet de la pénicilline. Compt. rend. Soc. Biol., **143**, 286–288.

87. TULASNE, R. 1949 Sur une evolution du Proteus vers des microcolonies et des formes inframicroscopiques sous l'effet de la pénicilline. Compt. rend. Soc. Biol., **143**, 289–291.
88. TULASNE, R. 1950 Quelques données nouvelles sur la formation et les caractères de culture des formes L du *Proteus vulgaris*. Compt. rend. Soc. Biol., **144**, 1200–1203.
89. TULASNE, R. Personal communication.
90. TULASNE, R., AND VENDRELY, R. 1947 Demonstration of bacterial nuclei with ribonuclease. Nature, **160**, p. 225.
91. VAN ROOYEN, C. E. 1936 The biology, pathogenesis and classification of *Streptobacillus moniliformis*. J. Path. Bact., **43**, 455–472.
92. WAHLEN, J. G., AND ALMADEN, P. J. 1939 The megalomorphic phase of bacteria. J. Infectious Dis., **65**, 147–155.
93. WEINBERGER, H. J., MADOFF, S., AND DIENES, L. 1950 The properties of L forms isolated from Salmonella and the isolation of L forms from Shigella. J. Bact., **59**, 765–775.
94. WINGE, O. 1935 On haplophase and diplophase in some saccharomycetes. C. R. Lab. Carlsberg, Ser. physiol., **21**, 77–112.